Inventor: Schwiebert et al.
Title: Methods and Compositions for P2X Receptor Calcium Entry Channels and Other Calcium Entry Mechanisms
Docket No.: 21085.0044U3
REPLACEMENT SHEET
Sheet 1 of 51

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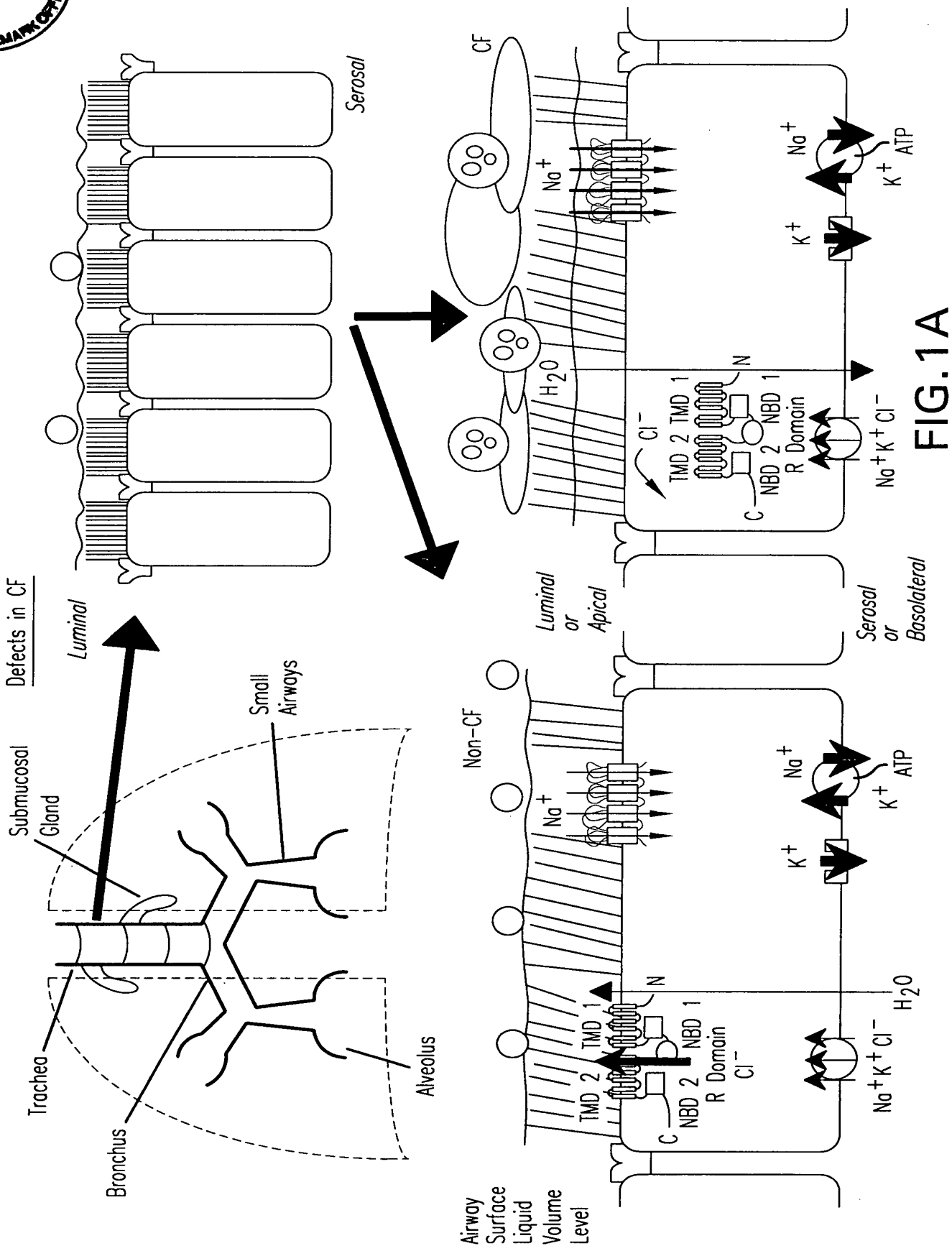


FIG. 1A

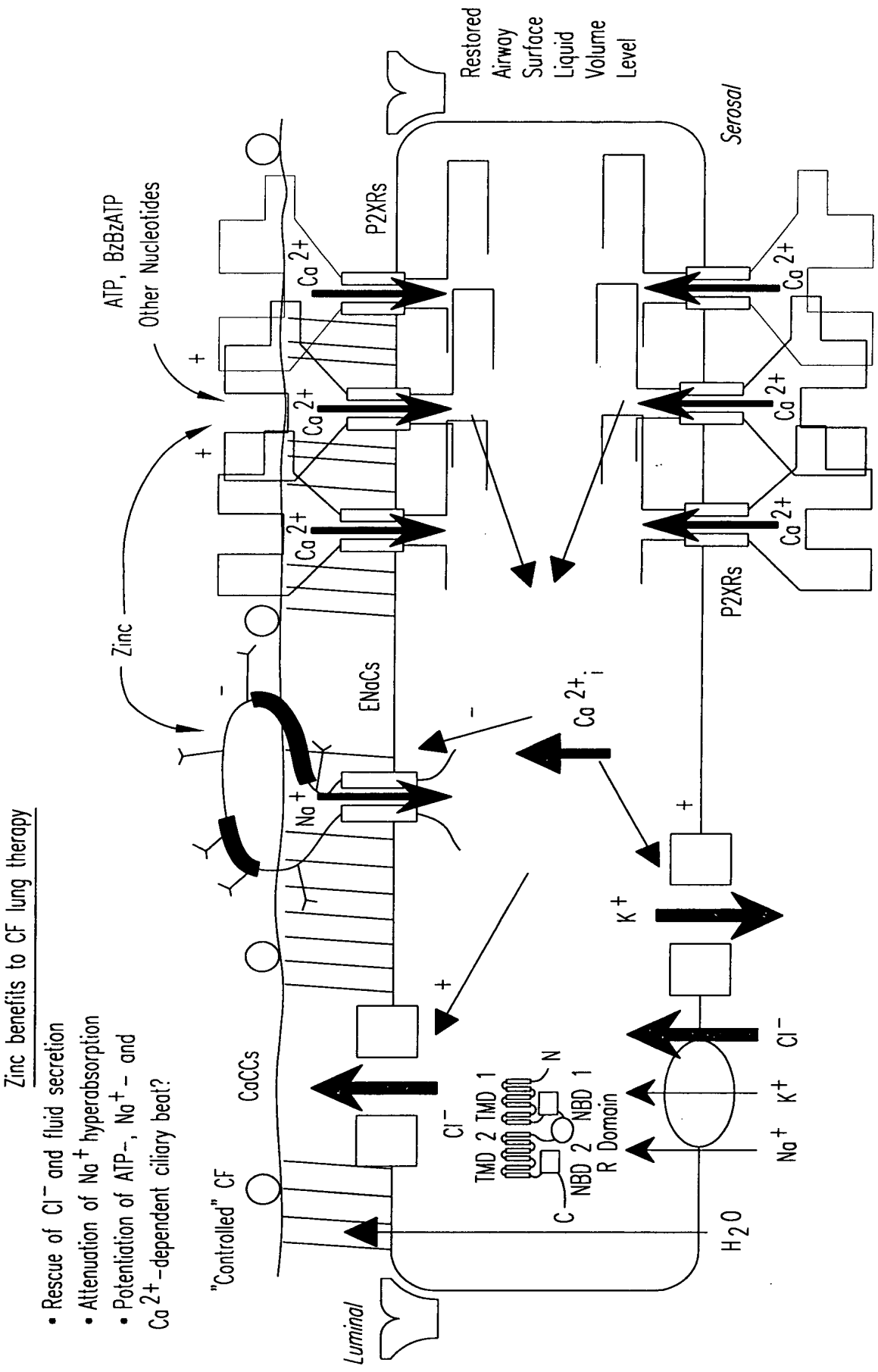

Zinc benefits to CF lung therapy
• Rescue of Cl⁻ and fluid secretion
• Attenuation of Na⁺ hyperabsorption
• Potentiation of ATP-, Na⁺- and Ca 2+-dependent ciliary beat?
Restored Airway Surface Liquid Volume Level
Serosal
ATP, BzBzATP
Other Nucleotides
P2XRs
Ca 2+
Zinc
ENaCs
Na+
Ca 2+ i
K+
"Controlled" CF
CaCCs
Cl⁻
TMD 2 TMD 1
N
NBD 2 NBD 1
R Domain
C
H2O
Na+ K+ Cl⁻
Luminal

FIG. 1B

Zinc as an anti-inflammatory for CF and other airway diseases such as asthma and common cold

- Zinc in a solution-based formulation enters the cell as free ionic zinc and inhibits Zinc NFkappaB activation

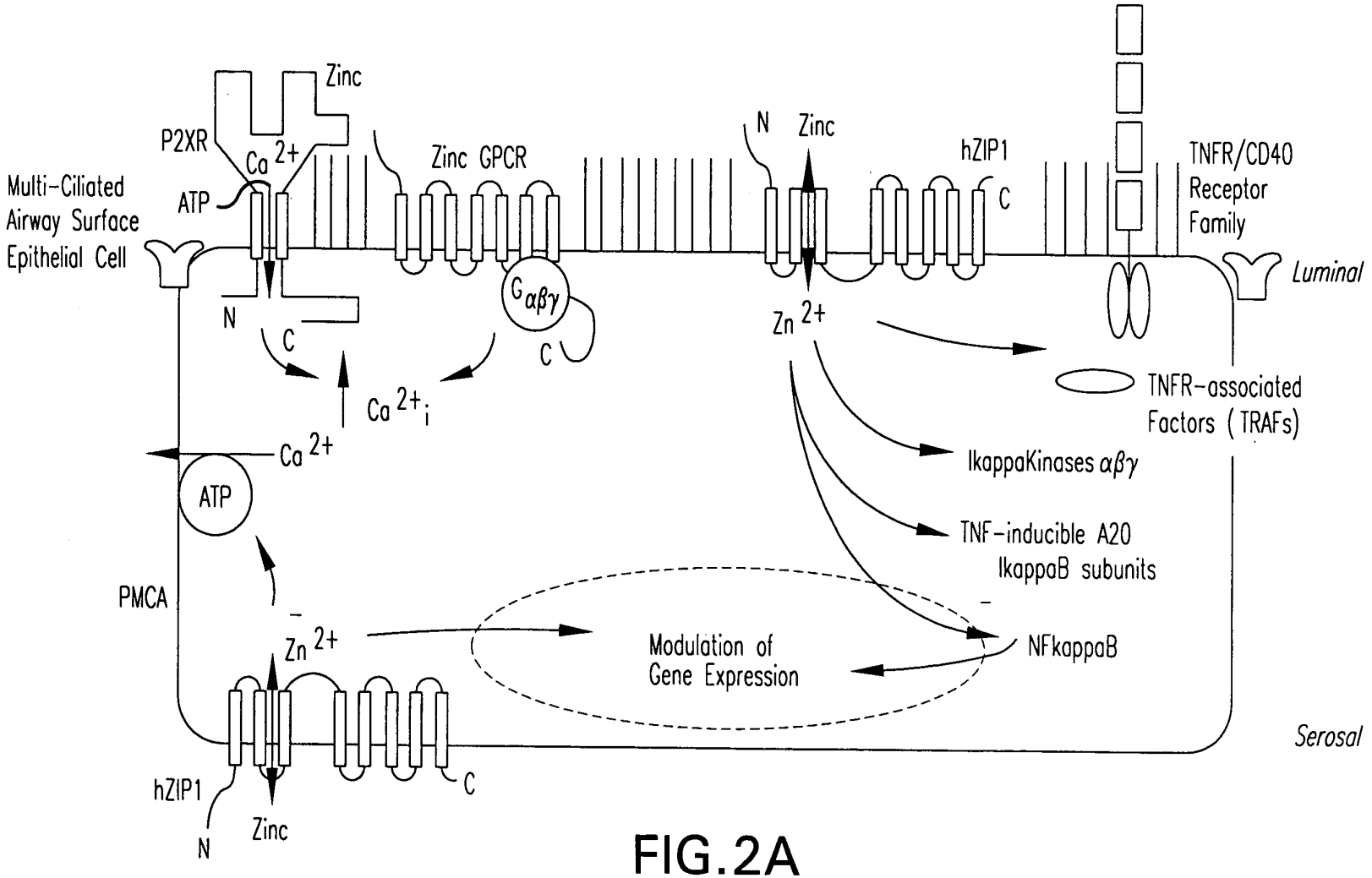


FIG.2A

Zinc as an anti-microbial for CF and other airway and GI diseases caused by bacterial pathogens

- Zinc in a solution-based formulation competitively inhibits the metal scavenging system of a bacterium.

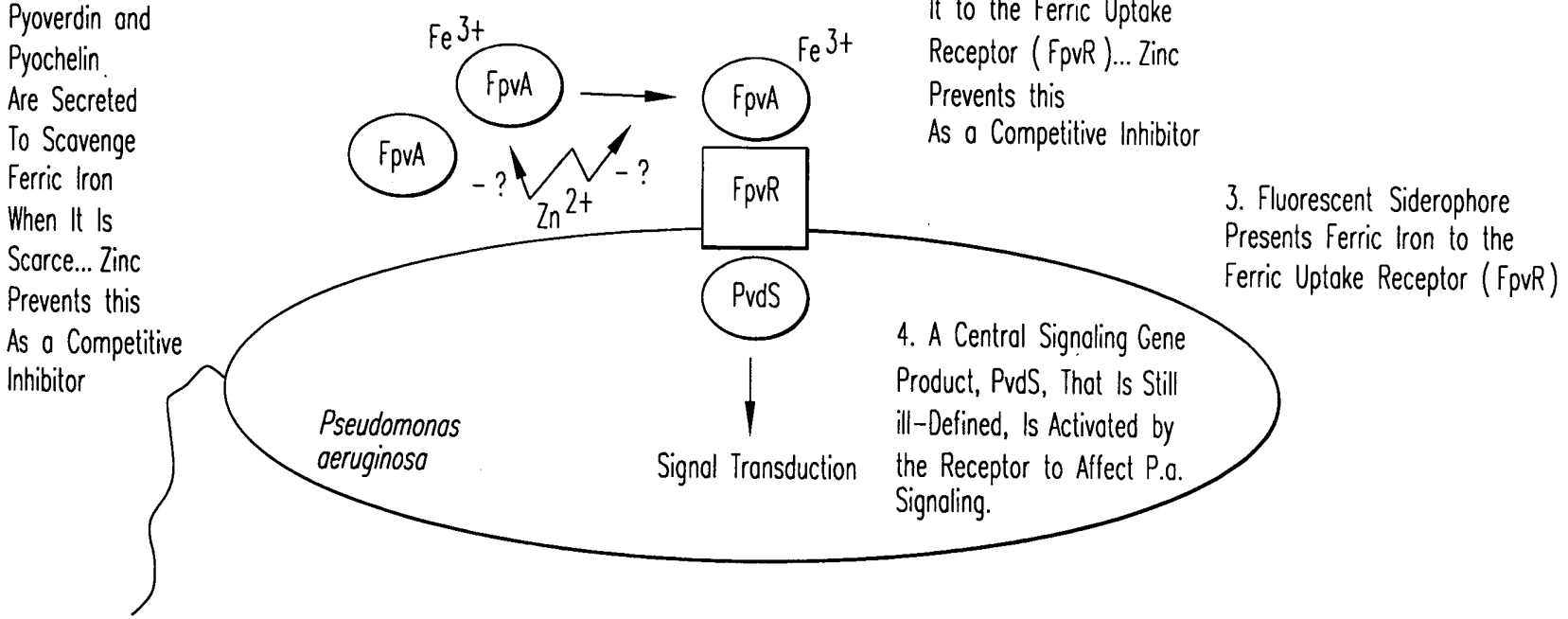


FIG.2B

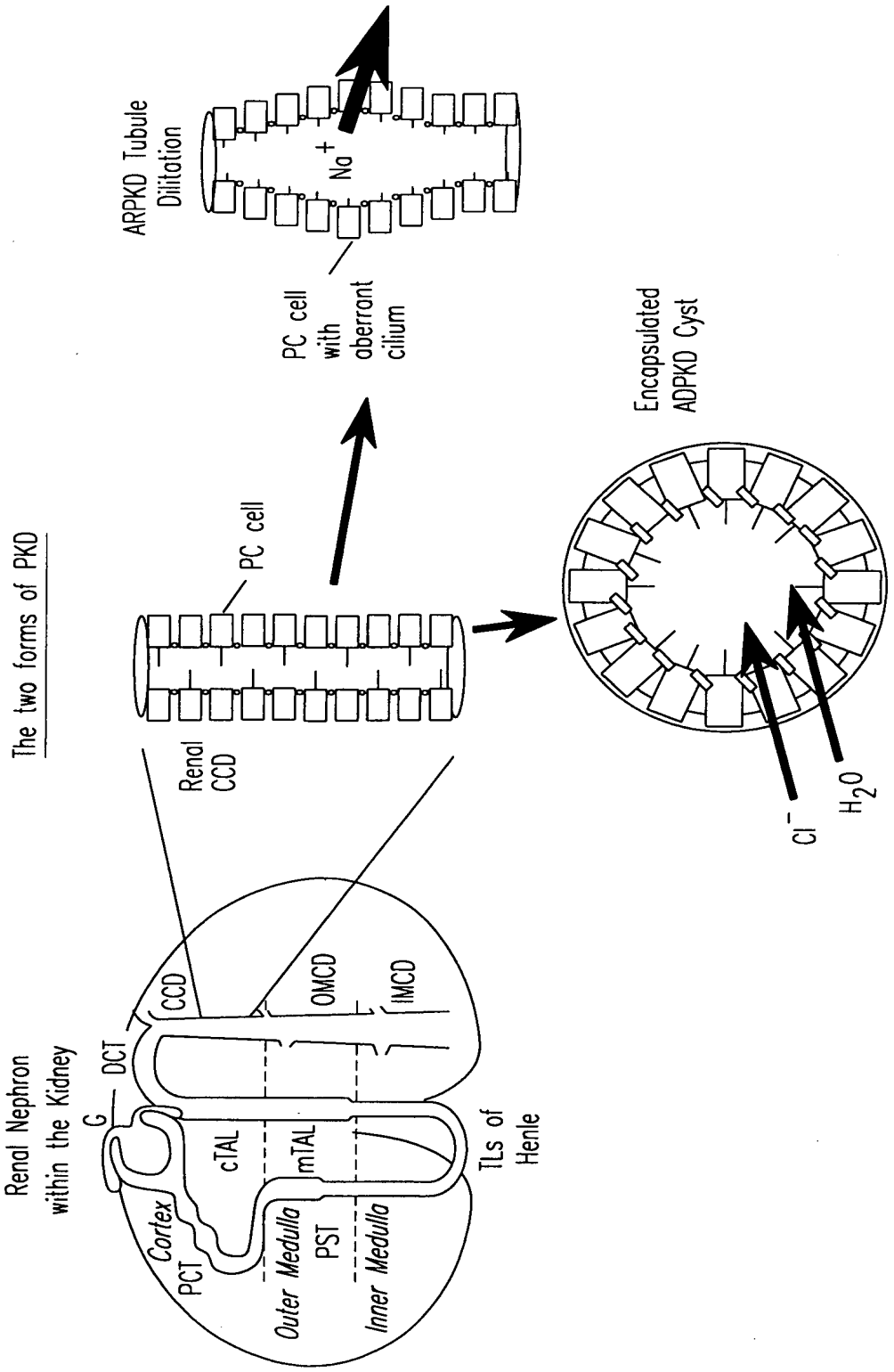

The two forms of PKD
Renal Nephron within the Kidney
G
DCT
CCD
OMCD
IMCD
cTAL
mTAL
TLs of Henle
Cortex
PCT
Outer Medulla
PST
Inner Medulla
Renal CCD
PC cell
PC cell with aberrant cilium
ARPKD Tubule Dilitation
Na^+
Encapsulated ADPKD Cyst
Cl^-
H_2O

FIG.3A

Zinc benefits to PKD therapy and therapy of other renal hypertensive disorders

- Direct inhibition of Na^+ hyperabsorption
- Stimulation of P2XR Ca^{2+} entry channels "alternative" to cilium-derived Ca^{2+} entry

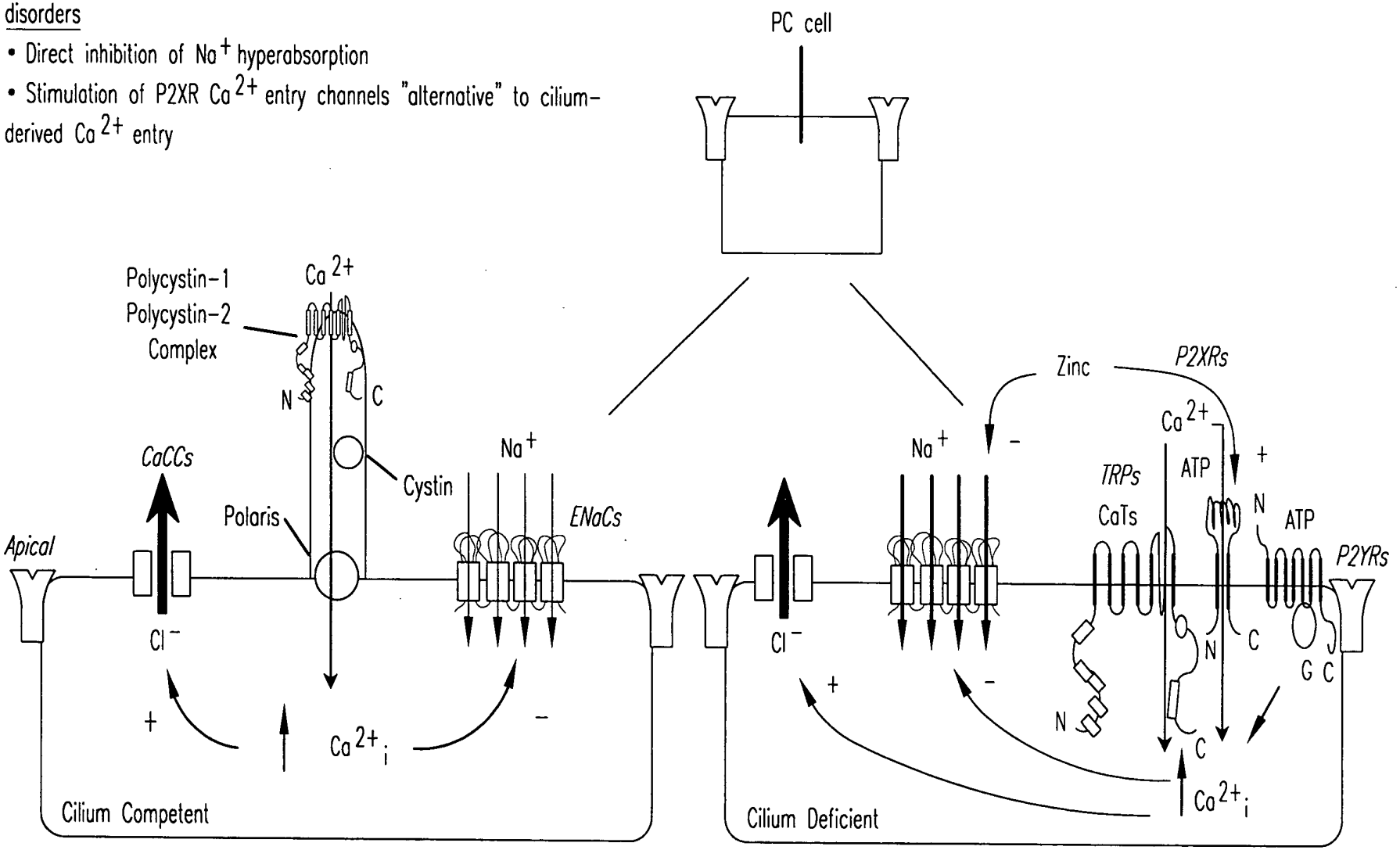


FIG.3B

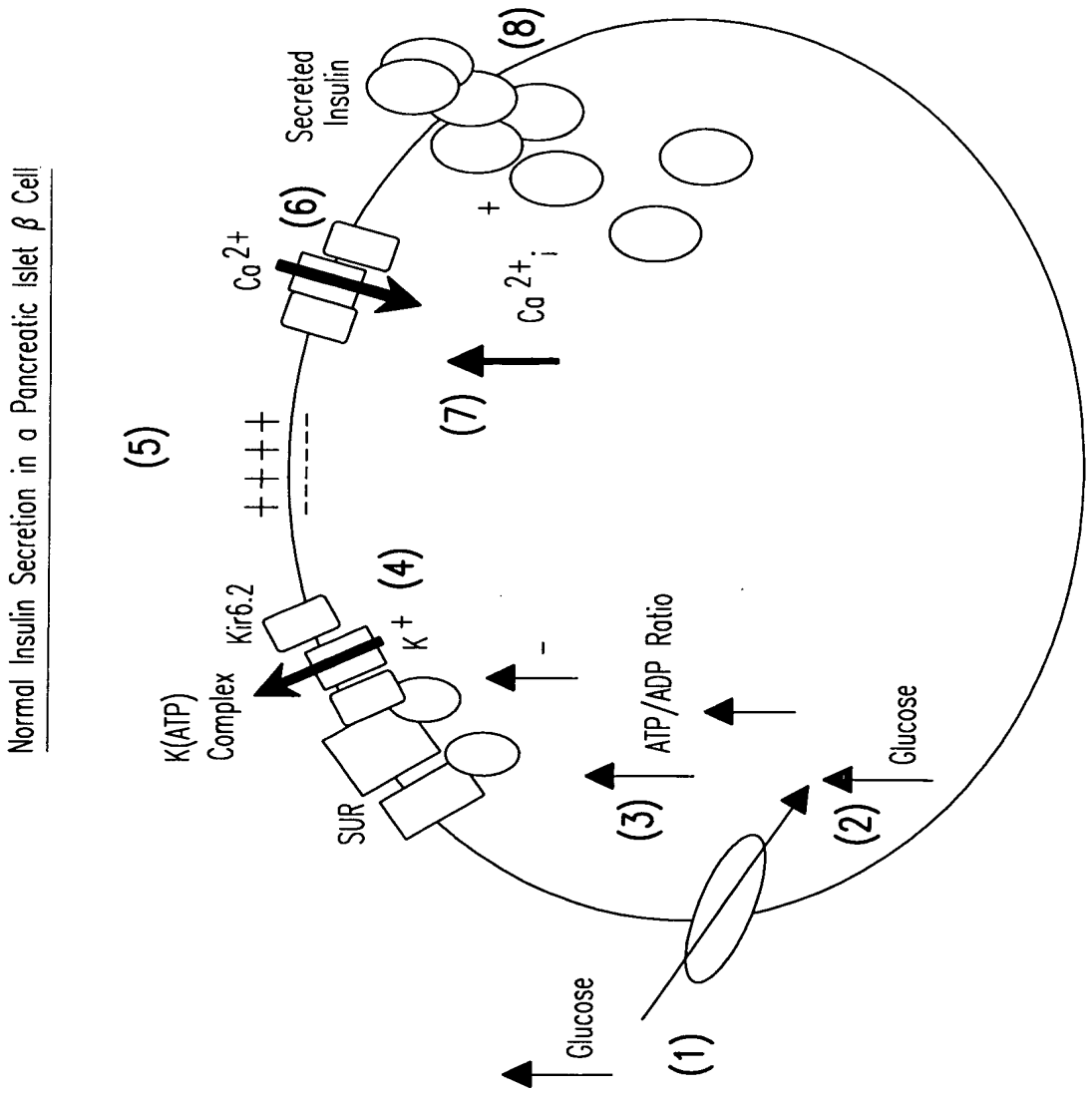

Normal Insulin Secretion in a Pancreatic Islet β Cell
Glucose
(1)
(2)
Glucose
(3)
ATP/ADP Ratio
(4)
K+
(5)
SUR
Kir6.2
K(ATP) Complex
(6)
Ca 2+
(7)
Ca 2+ i
(8)
Secreted Insulin

FIG.4A

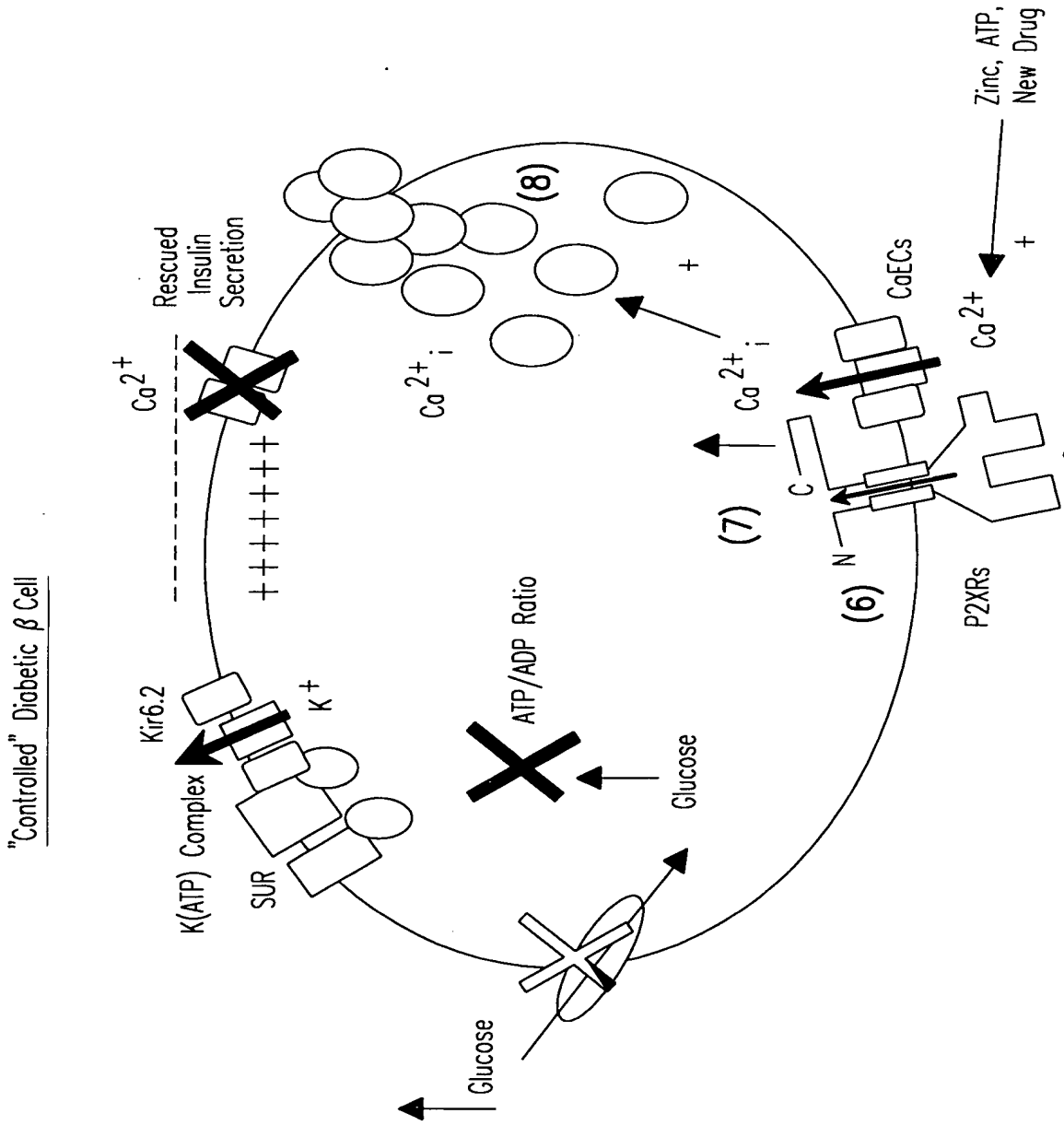

"Controlled" Diabetic β Cell
Rescued Insulin Secretion
Ca^{2+}
Kir6.2
K^+
K(ATP) Complex
SUR
ATP/ADP Ratio
Glucose
Glucose
Ca^{2+}_i
(8)
Ca^{2+}_i
(7)
C
N
(6)
CaECs
Ca^{2+}
P2XRs
Ca^{2+}
Zinc, ATP, New Drug
+

FIG.4B

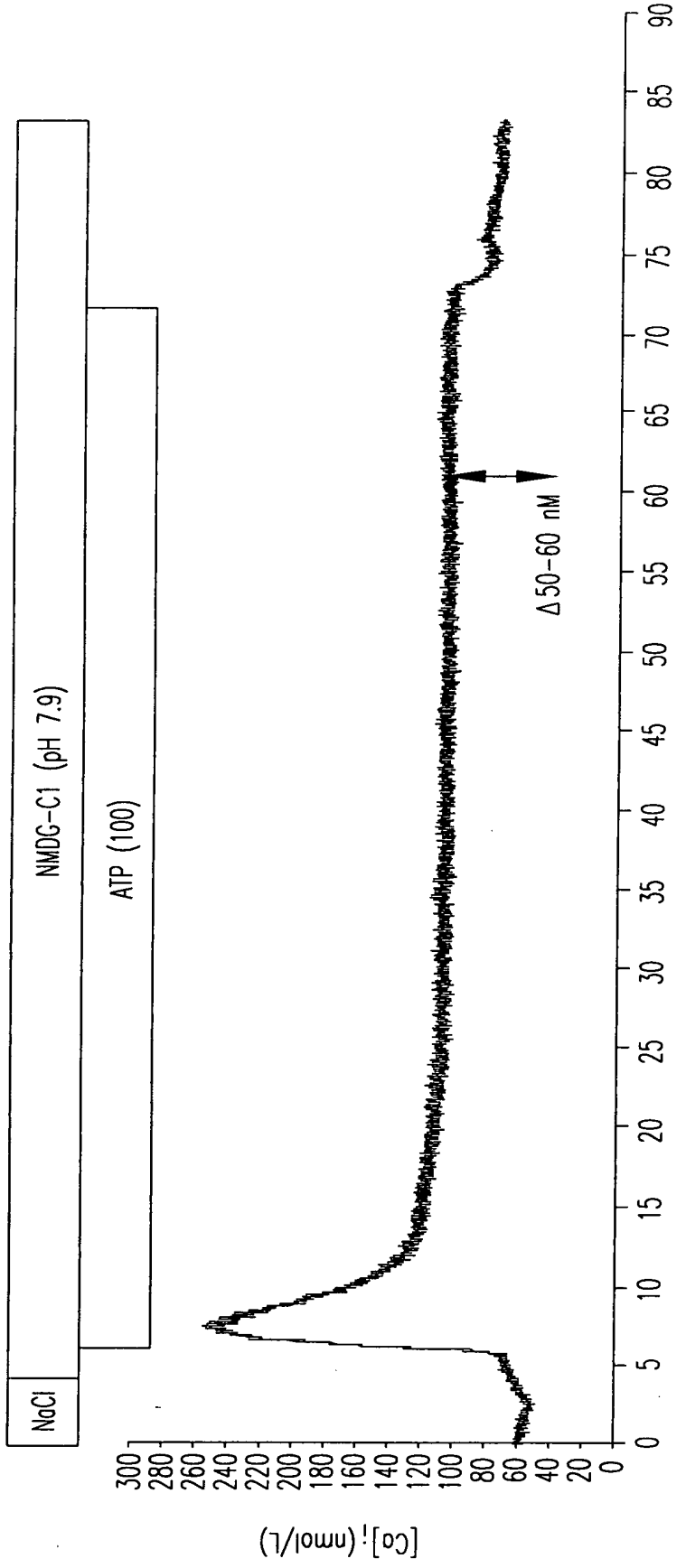

NaCl
NMDG-Cl (pH 7.9)
ATP (100)
Δ50-60 nM
[Ca]i (nmol/L)
Time (min)
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90

FIG.5A-1

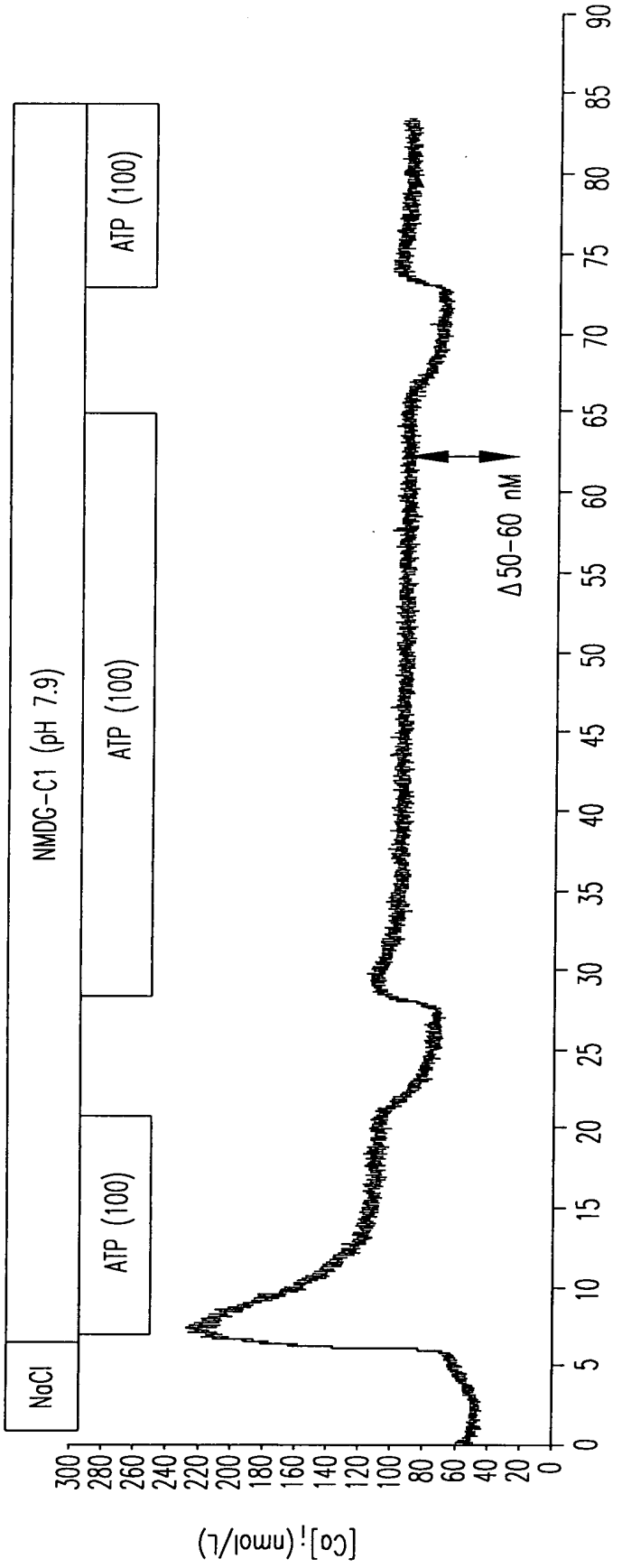
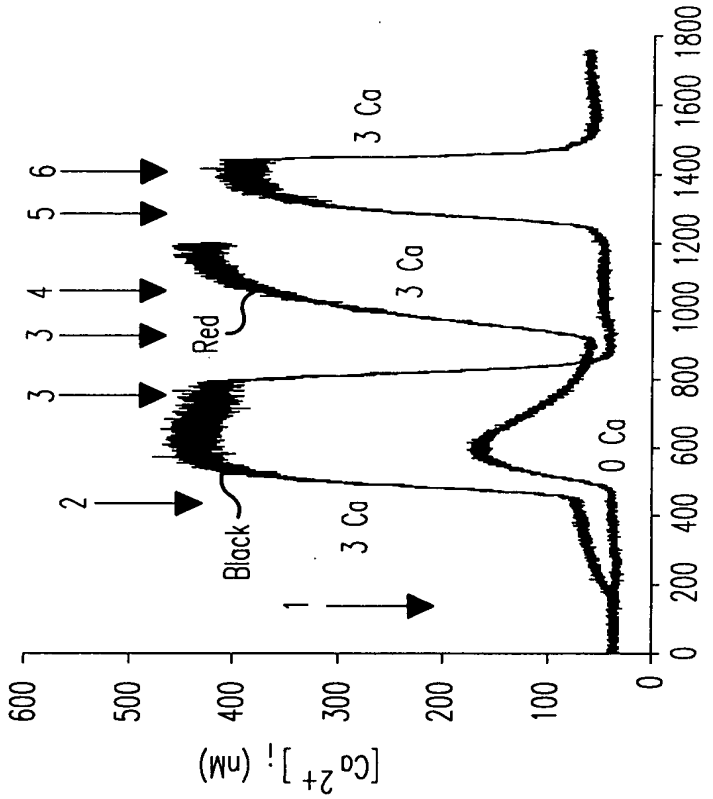
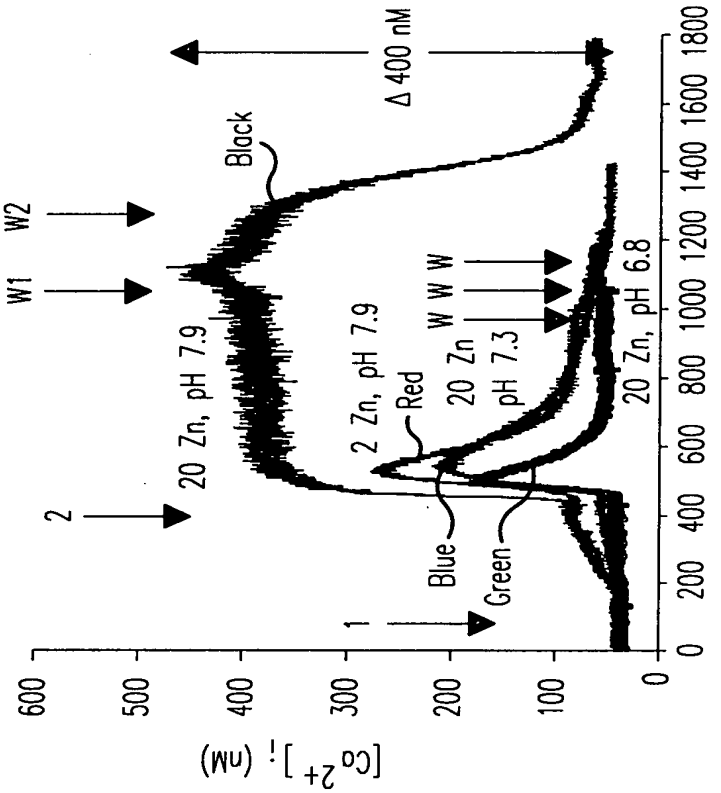

NaCl
NMDG-C1 (pH 7.9)
ATP (100)
ATP (100)
ATP (100)
Δ50-60 nM
[Ca]i (nmol/L)
Time (min)
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90

FIG.5A-2



Black = 20 Zn, pH 7.9 plus Extracellular Ca^{2+}
Red = 20 Zn, pH 7.9, 0 Extracellular Ca^{2+}, then, add back 3 mM Ca^{2+}



Black = 100 ATP, 20 Zn, pH 7.9
Red = 100 ATP, 2 Zn, pH 7.9
Blue = 100 ATP, 20 Zn, pH 7.3
Green = 100 ATP, 20 Zn, pH 6.8

FIG.5B

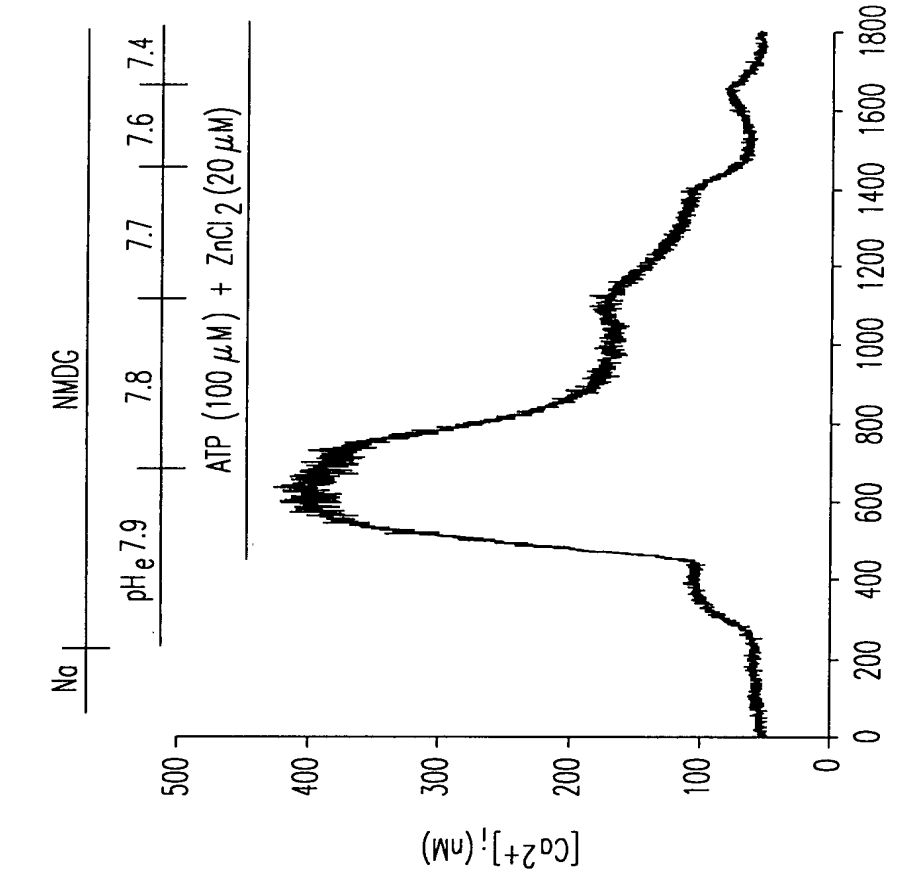

NMDG
Na
pH e 7.9
7.8
7.7
7.6
7.4
ATP (100 μM) + ZnCl 2 (20 μM)
[Ca2+]i (nM)
500
400
300
200
100
0
0 200 400 600 800 1000 1200 1400 1600 1800
Time (s)

FIG.5D

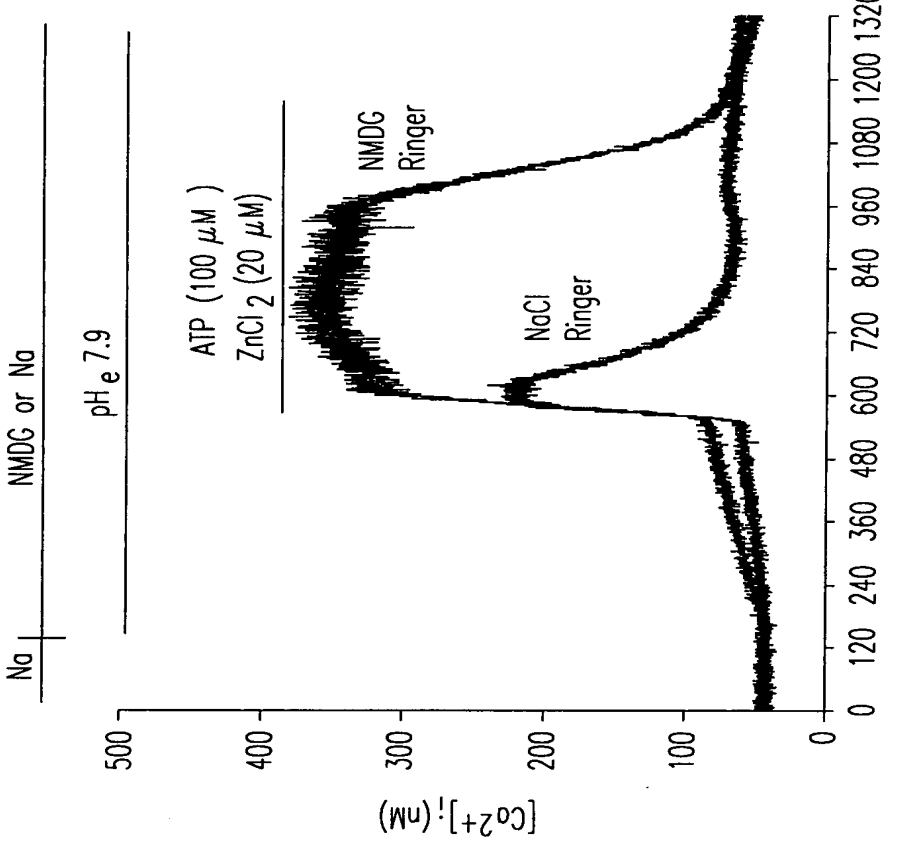

Na
NMDG or Na
pH e 7.9
ATP (100 μM)
ZnCl 2 (20 μM)
NMDG
Ringer
NaCl
Ringer
[Ca2+]i (nM)
500
400
300
200
100
0
0 120 240 360 480 600 720 840 960 1080 1200 1320
Time (s)

FIG.5C

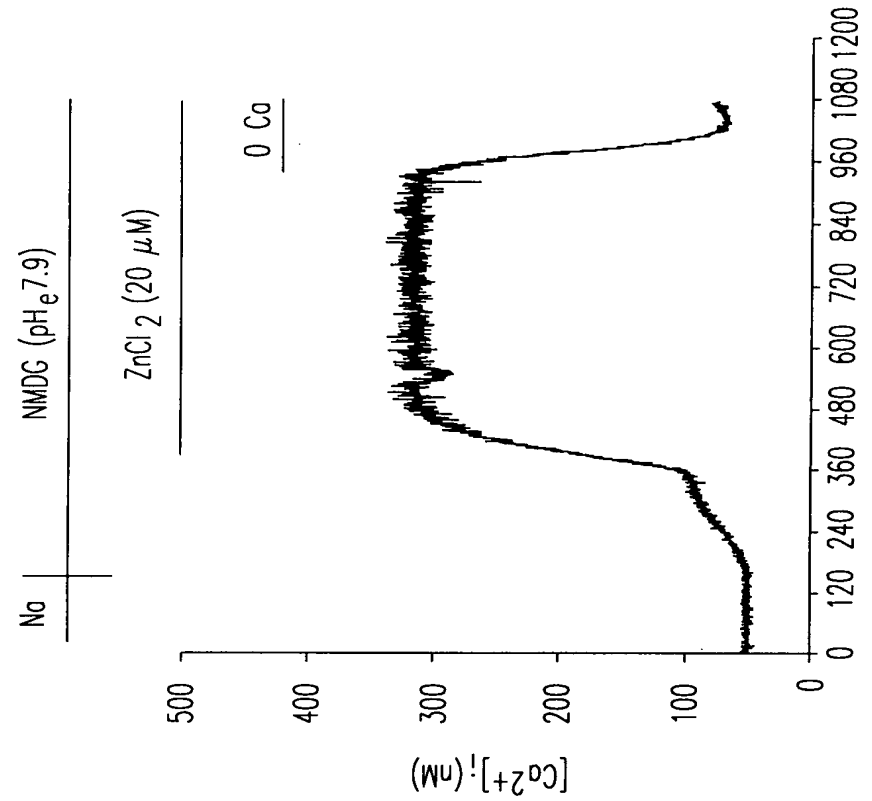

Na
NMDG (pH$_e$7.9)
ZnCl 2 (20 μM)
0 Ca
[Ca2+]$_i$ (nM)
500
400
300
200
100
0
0 120 240 360 480 600 720 840 960 1080 1200
Time (s)

FIG.5F

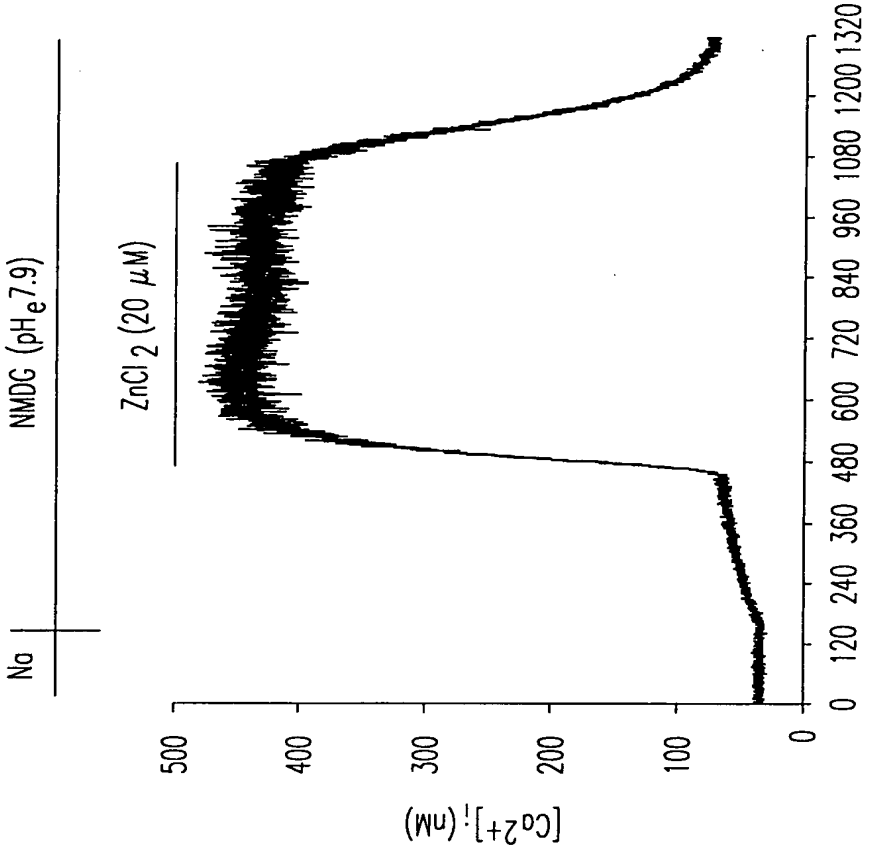

Na
NMDG (pH$_e$7.9)
ZnCl 2 (20 μM)
[Ca2+]$_i$ (nM)
500
400
300
200
100
0
0 120 240 360 480 600 720 840 960 1080 1200 1320
Time (s)

FIG.5E

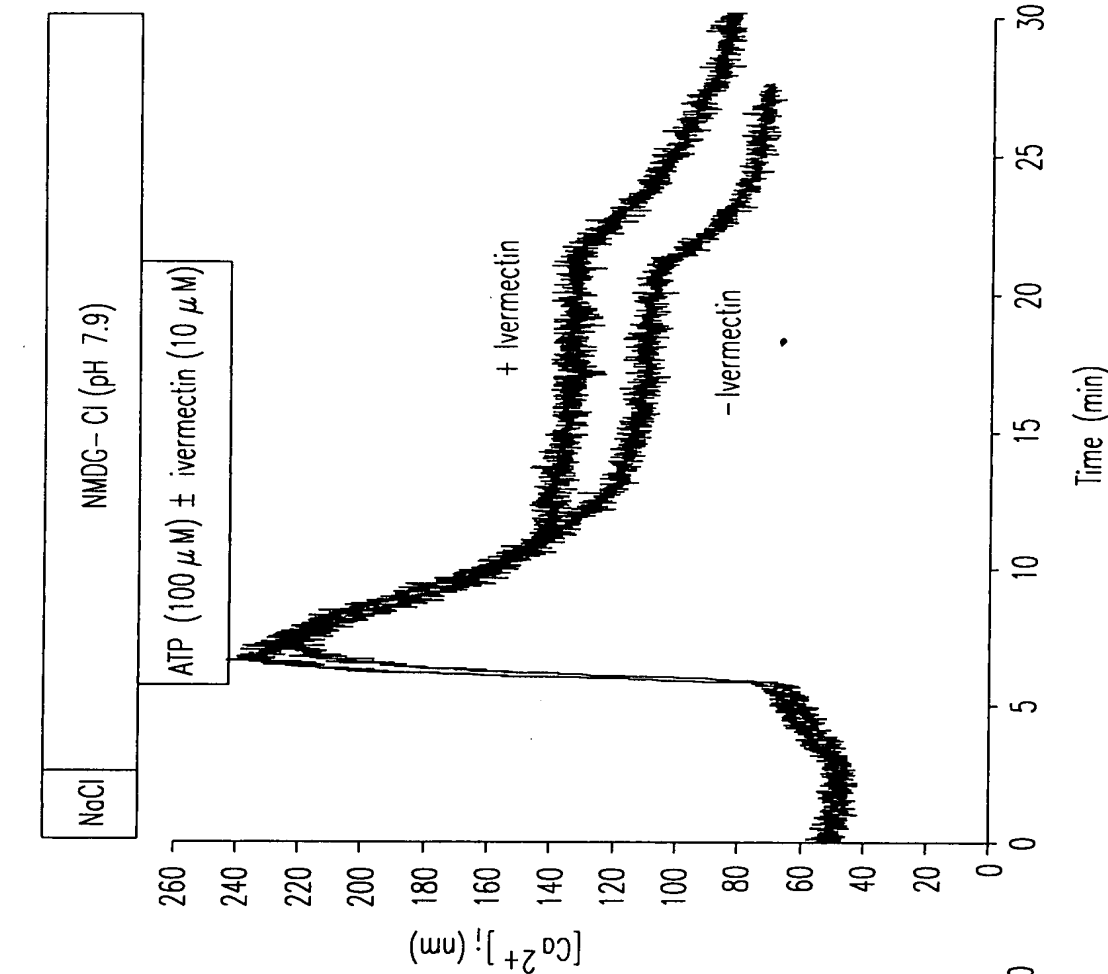

NaCl
NMDG- Cl (pH 7.9)
ATP (100 μM) ± ivermectin (10 μM)
+ Ivermectin
- Ivermectin
$[Ca^{2+}]_i$ (nm)
Time (min)
NMDG (pH_e 7.9)
$MnCl_2$
$ZnCl_2$ alone or
+ivermectin or
+ivermectin+ATP
Zinc
Zinc + Iver
Zinc + Iver + ATP
% of Fura-2 fluorescence (359 nm)
Time (sec)

FIG.6A

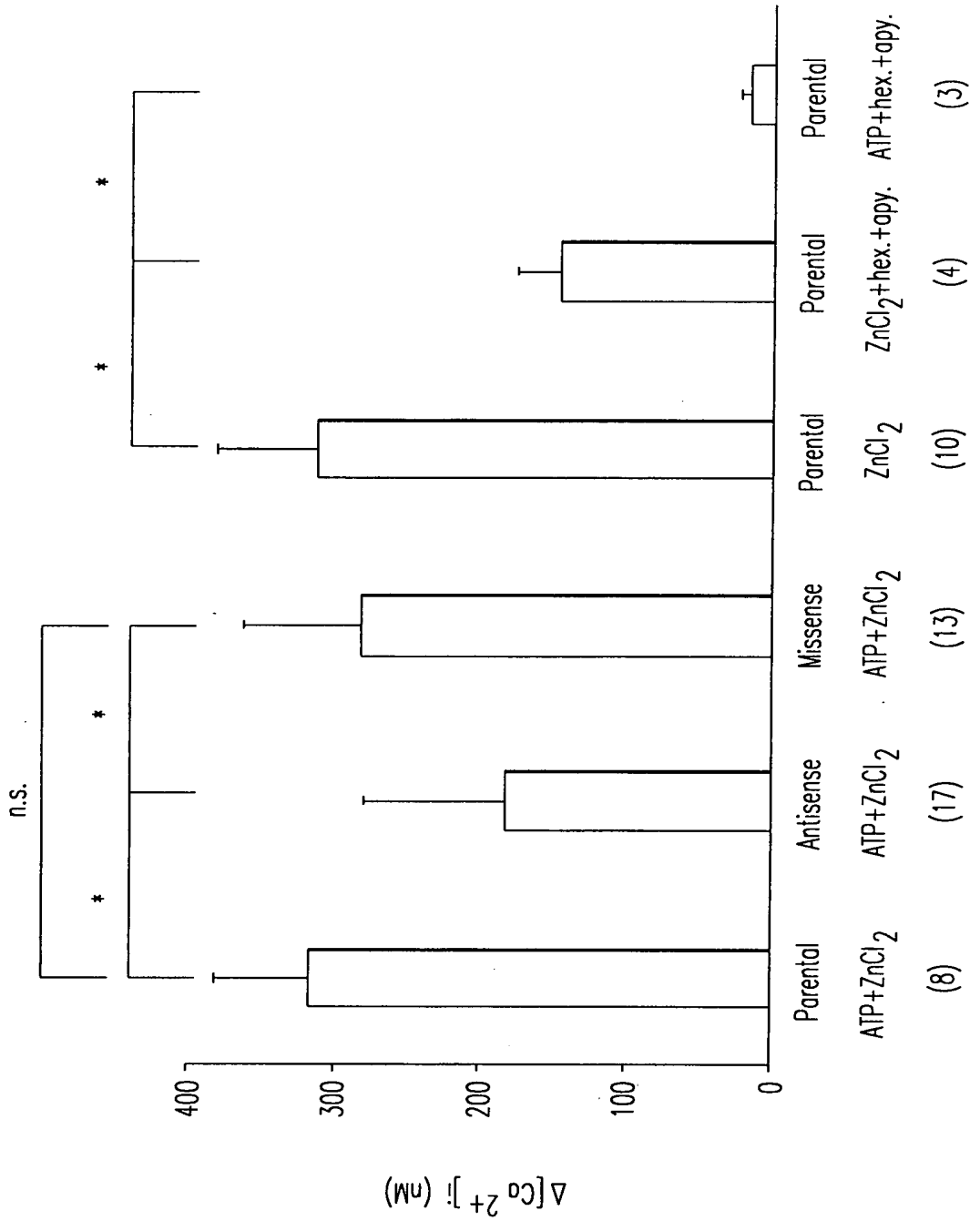

Δ[Ca 2+]i (nM)
400
300
200
100
0
n.s.
*
*
*
*
Parental
Antisense
Missense
Parental
Parental
Parental
ATP+ZnCl2
ATP+ZnCl2
ATP+ZnCl2
ZnCl2
ZnCl2+hex.+apy.
ATP+hex.+apy.
(8)
(17)
(13)
(10)
(4)
(3)

FIG.6B

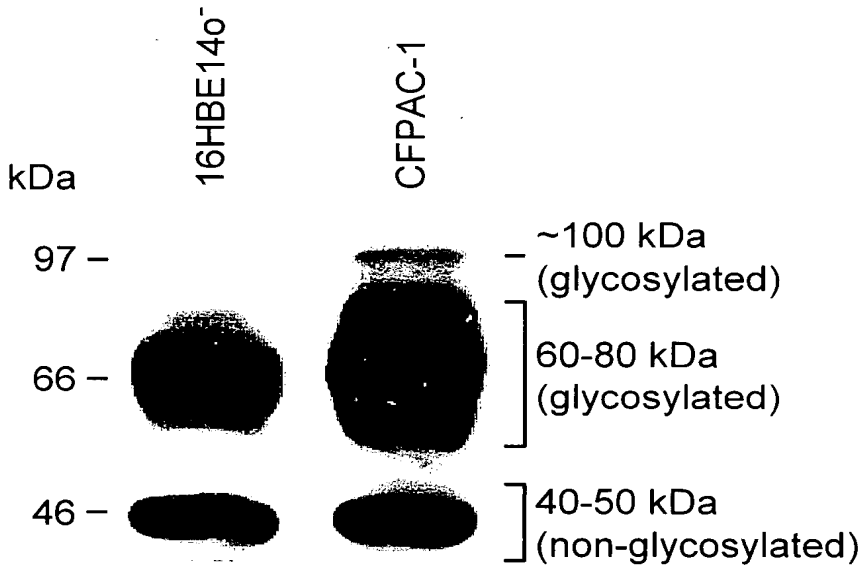

16HBE14o-
CFPAC-1
kDa
97 –
66 –
46 –
~100 kDa
(glycosylated)
60-80 kDa
(glycosylated)
40-50 kDa
(non-glycosylated)

FIG.7A

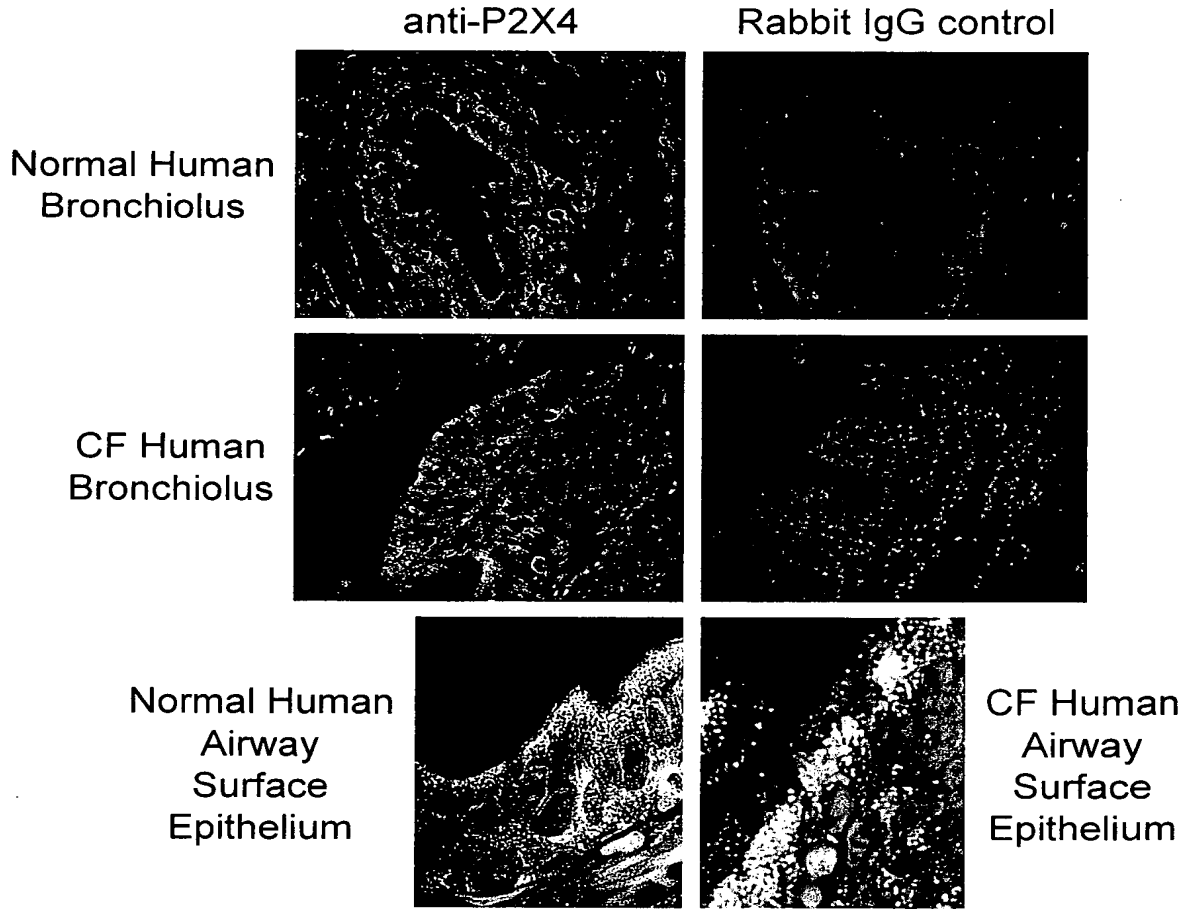

anti-P2X4
Rabbit IgG control
Normal Human Bronchiolus
CF Human Bronchiolus
Normal Human Airway Surface Epithelium
CF Human Airway Surface Epithelium

FIG.7B

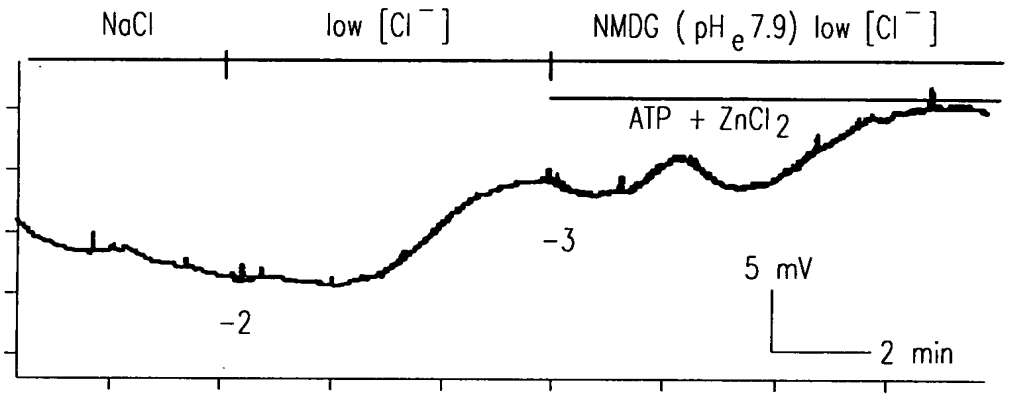


FIG.8A

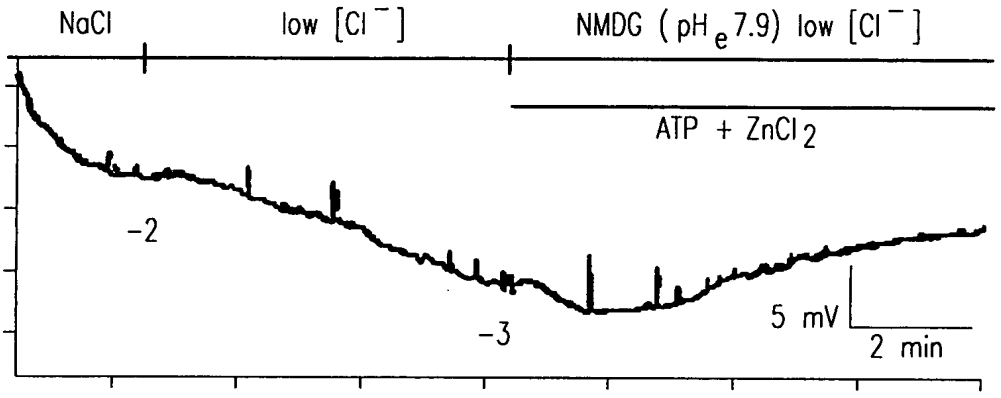


FIG.8B

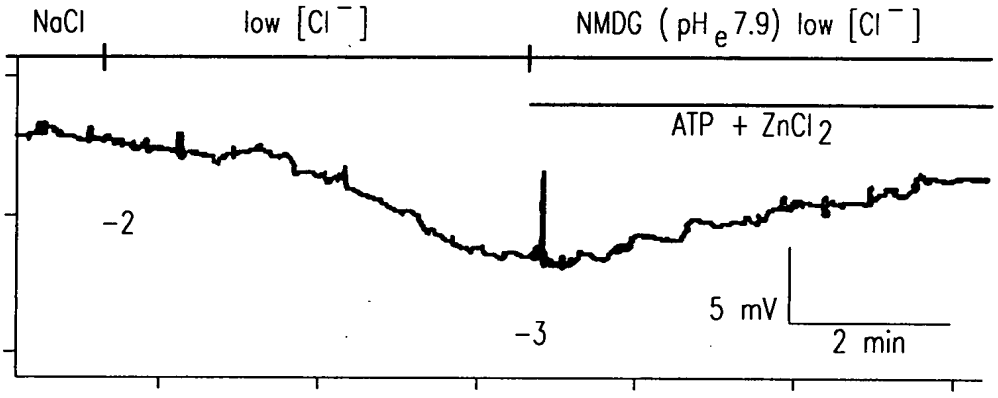


FIG.8C

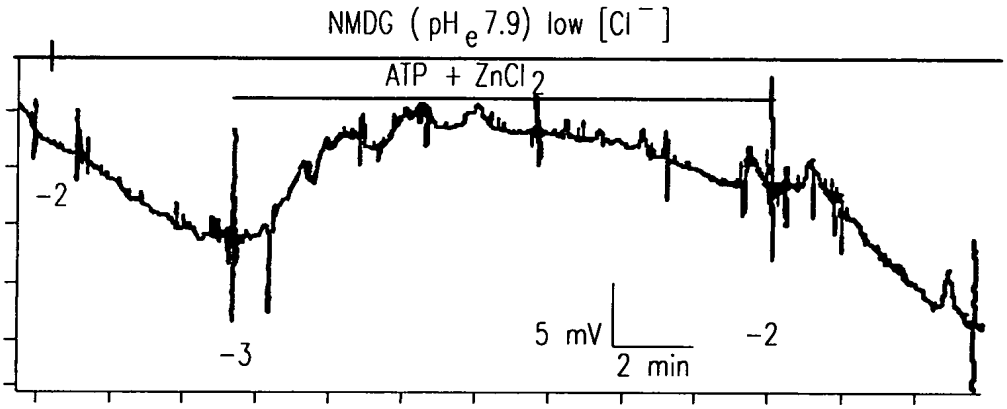

NMDG (pH e 7.9) low [Cl ⁻]
ATP + ZnCl 2
-2
-3
5 mV
2 min
-2

FIG.8D

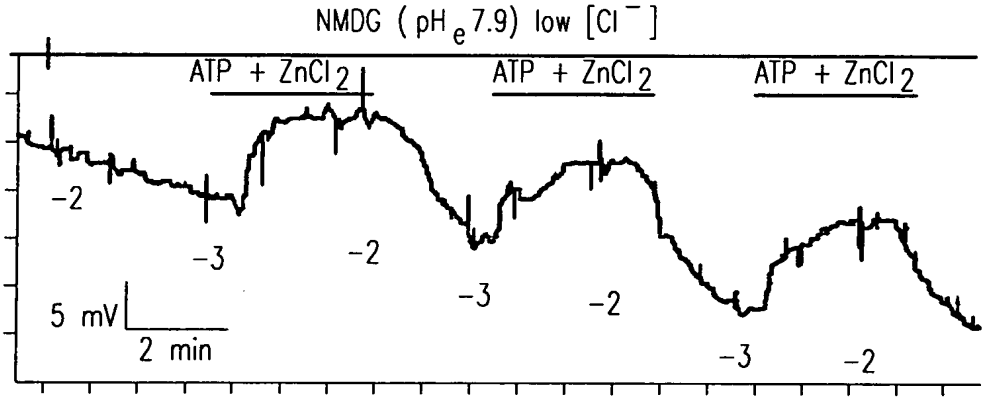

NMDG (pH e 7.9) low [Cl ⁻]
ATP + ZnCl 2
ATP + ZnCl 2
ATP + ZnCl 2
-2
-3
-2
-3
-2
-3
-2
5 mV
2 min

FIG.8E

Transepithelial Nasal Potential Difference Values of Control, Δ508 CF and Bitransgenic CF Mice

	Control Cftr(+/−)	n	CF Cftr(ΔF508/ ΔF508)	n	Bitransgenic CF Cftr(−/−)	n
Starting point	−18.7 ± 6.5	19	−26.3 ± 7.2*	11	−26.1 ± 3.8*	14
Low $[Cl^-]_e$ (Na^+; pH:7.3)	−5.5 ± 1.5	8	+3.7 ± 1.6*	3	+4.8 ± 2.5*	7
ATP + $ZnCl_2$ (NMDG; pH:7.9)	−4.7 ± 1.8	6	−4.0 ± 2.0	3	−3.8 ± 2.0	12
Low $[Cl^-]_e$ (Na^+; pH:7.9)	−4.8 ± 2.0	6	+5.4 ± 2.8*	7	+6.7 ± 4.0*	3
ATP + $ZnCl_2$ (NMDG; pH:7.9)	−6.0 ± 1.4	2	−9.4 ± 1.6*#	8	−9.7 ± 3.1*&	3
Low $[Cl^-]_e$ (NMDG; pH:7.9)	−4.8 ± 3.3	5			+5.8 ± 1.9*	4
ATP + $ZnCl_2$ (NMDG; pH:7.9)	−5.7 ± 1.2	3			−10.2 ± 1.3*&	6
ATP alone (NMDG; pH:7.9)					−2.3 ± 1.0§	4
Low $[Cl^-]_e$ (NMDG; no added Ca^{2+}; pH:7.9)	−7.3 ± 0.6	3			+6.0 ± 0.8*	4
ATP + $ZnCl_2$ (NMDG; no added Ca^{2+}; pH:7.9)	−1.3 ± 0.6$	3			−2.0 ± 1.2$	4

FIG.8F

Inventor: Schwiebert et al.
Title: Methods and Compositions for P2X Receptor Calcium Entry Channels and Other Calcium Entry Mechanisms
Docket No.: 21085.0044U3
REPLACEMENT SHEET
Sheet 19 of 51

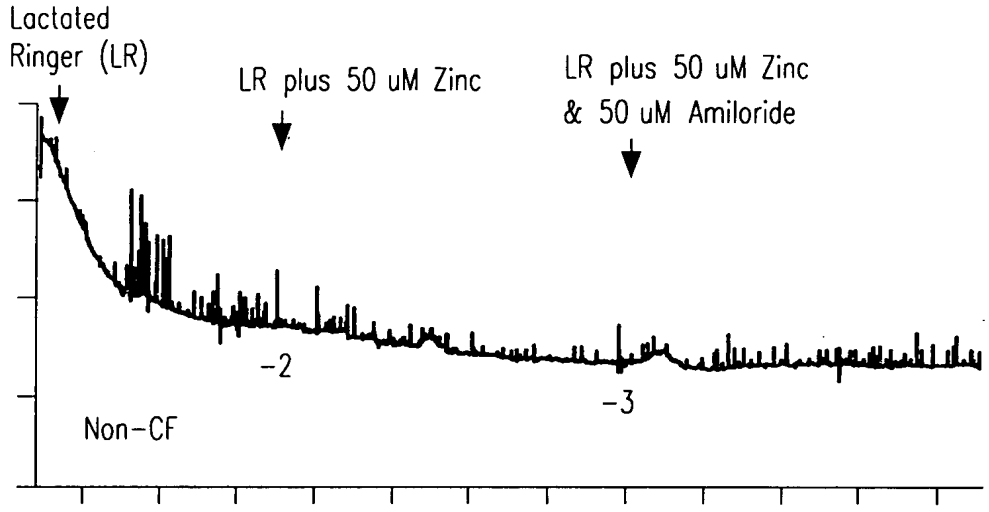

Lactated
Ringer (LR)
LR plus 50 uM Zinc
LR plus 50 uM Zinc
& 50 uM Amiloride
-2
-3
Non-CF

FIG.9A

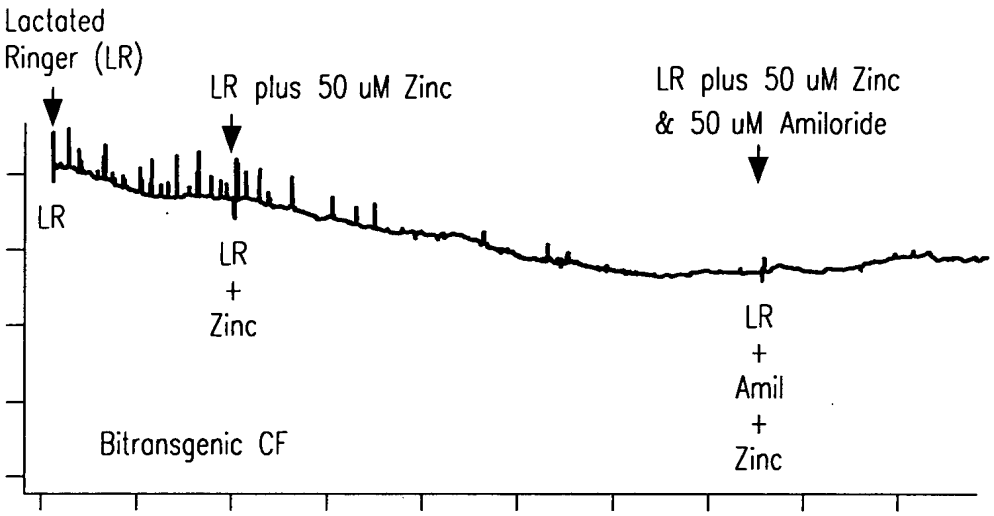

Lactated
Ringer (LR)
LR plus 50 uM Zinc
LR plus 50 uM Zinc
& 50 uM Amiloride
LR
LR
+
Zinc
LR
+
Amil
+
Zinc
Bitransgenic CF

FIG.9B

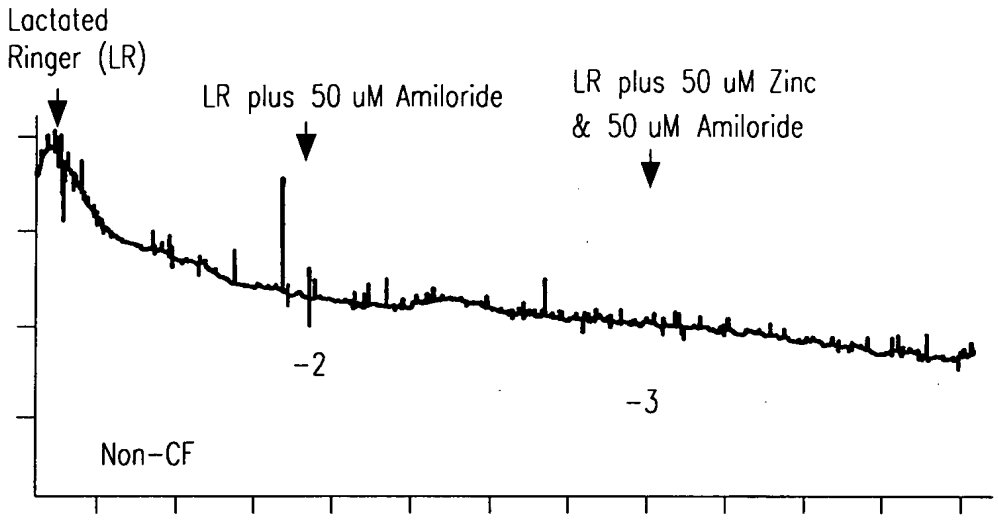

Lactated
Ringer (LR)
LR plus 50 uM Amiloride
LR plus 50 uM Zinc
& 50 uM Amiloride
-2
-3
Non-CF

FIG.9C

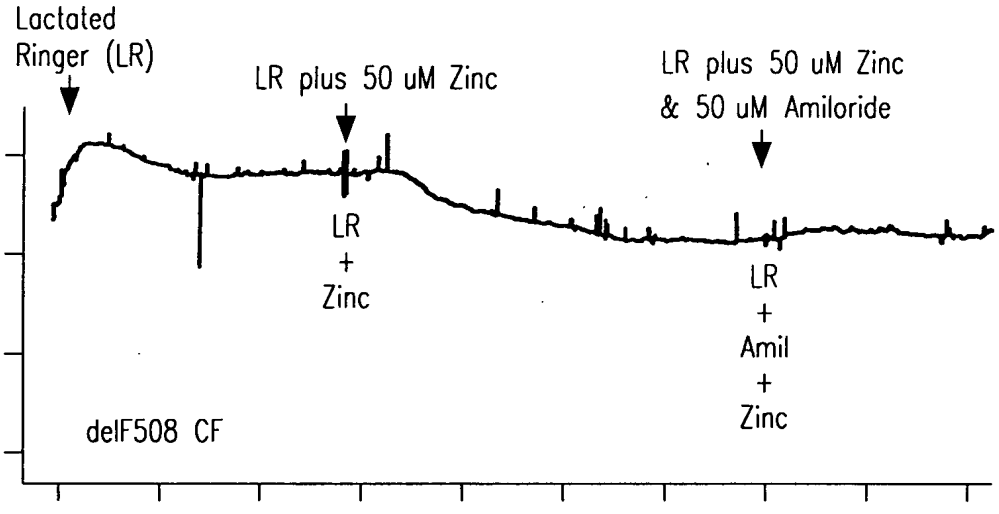

Lactated
Ringer (LR)
LR plus 50 uM Zinc
LR plus 50 uM Zinc
& 50 uM Amiloride
LR
+
Zinc
LR
+
Amil
+
Zinc
delF508 CF

FIG.9D

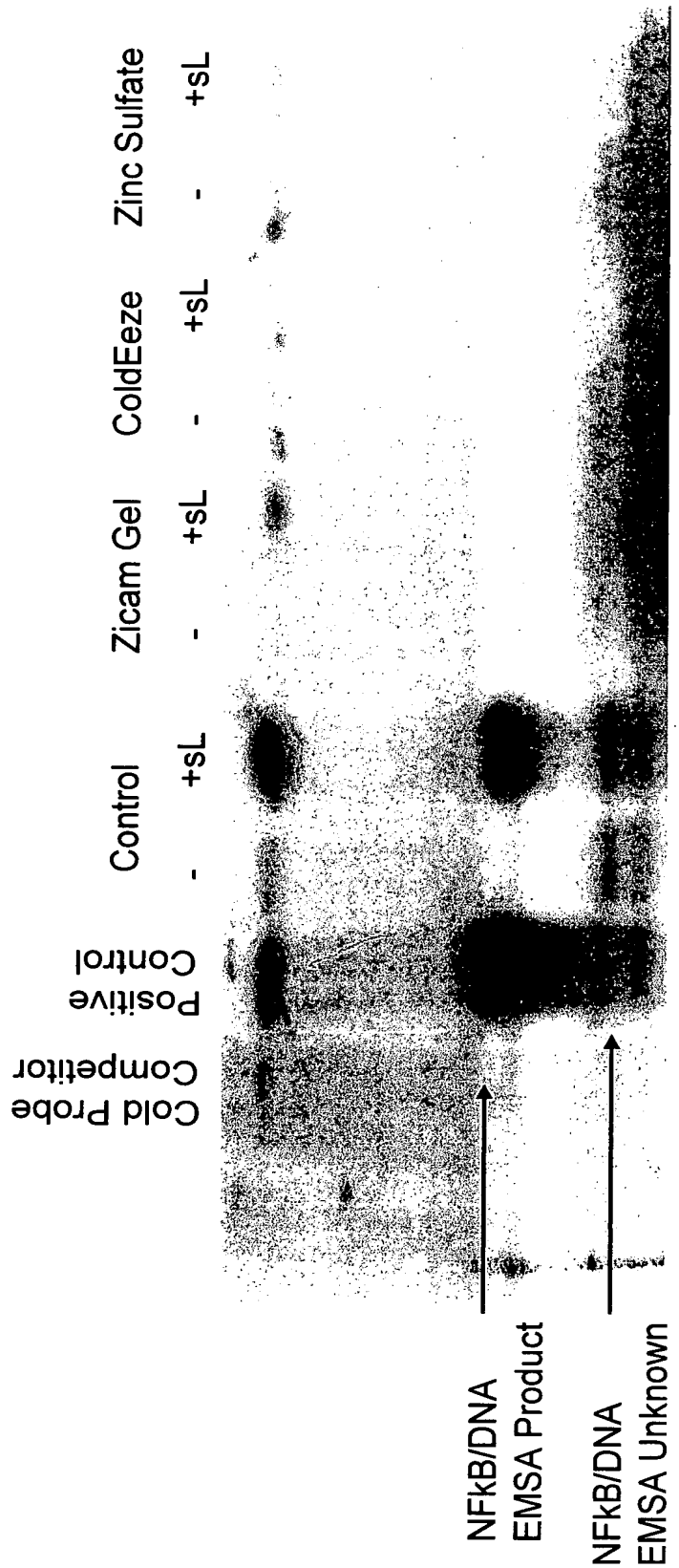

Cold Probe Competitor
Positive Control
Control
Zicam Gel
ColdEeze
Zinc Sulfate
-
+sL
-
+sL
-
+sL
-
+sL
NFkB/DNA EMSA Product
NFkB/DNA EMSA Unknown Secondary Product

FIG.10A

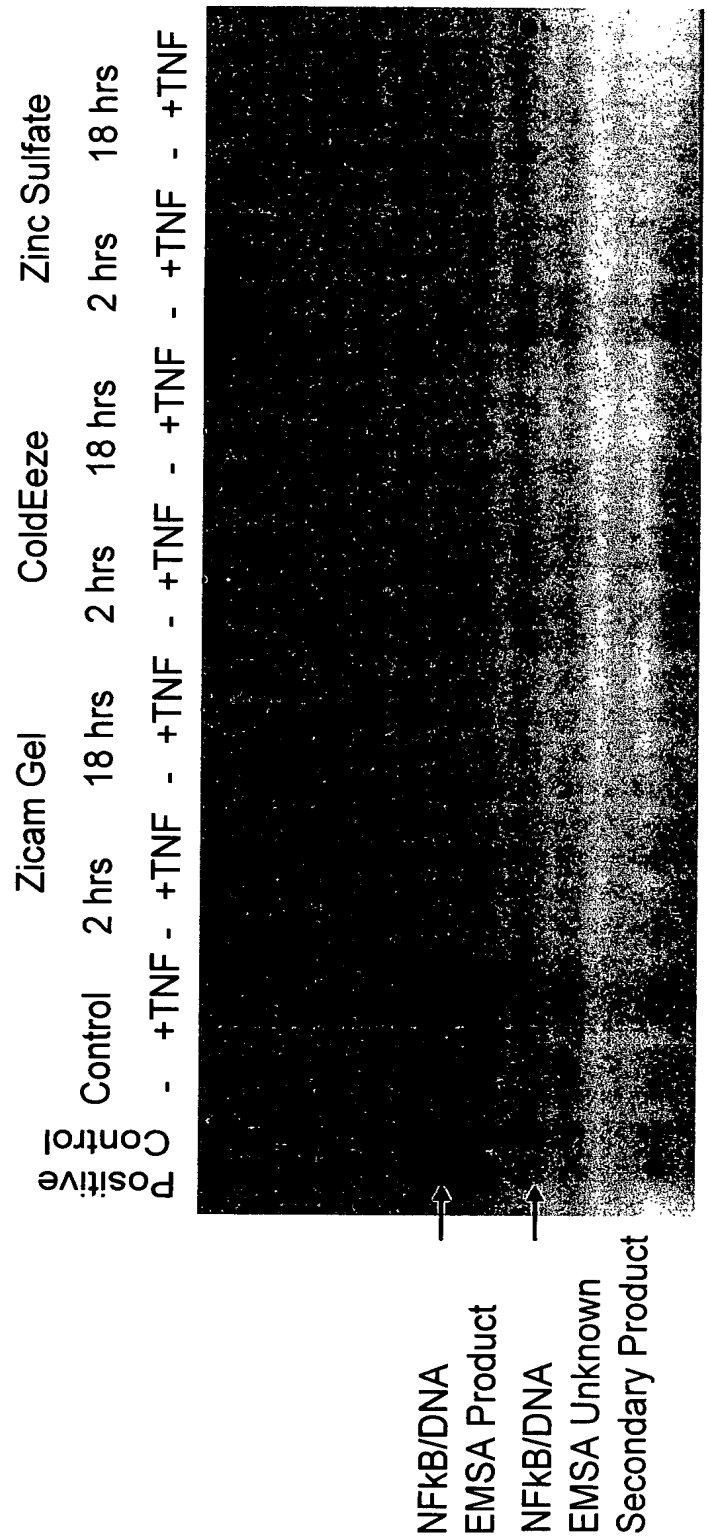

Positive Control
Zicam Gel
ColdEeze
Zinc Sulfate
- Control - +TNF
2 hrs - +TNF
18 hrs - +TNF
2 hrs - +TNF
18 hrs - +TNF
2 hrs - +TNF
18 hrs - +TNF
NFkB/DNA EMSA Product
NFkB/DNA EMSA Unknown Secondary Product

FIG.10B

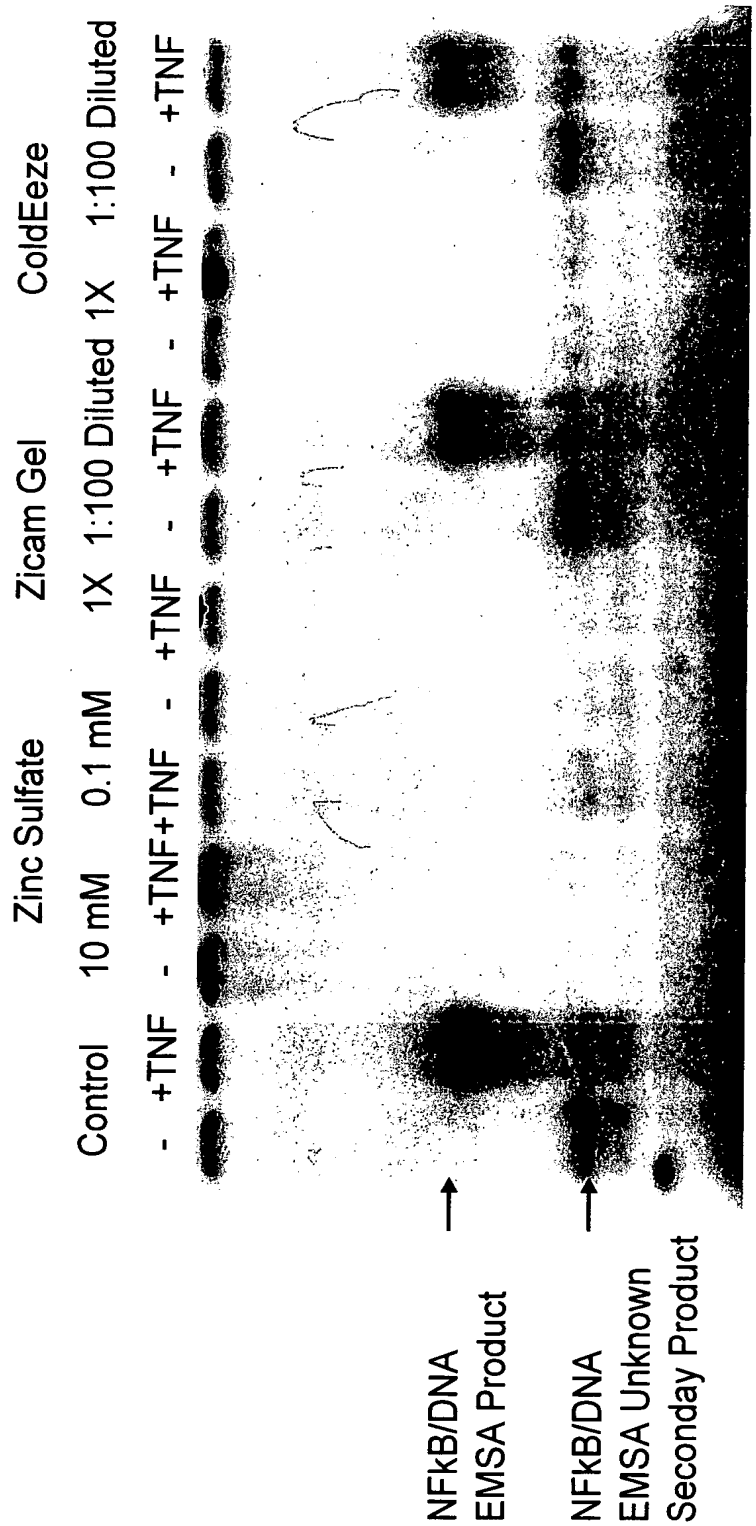

Zinc Sulfate
Zicam Gel
ColdEeze
Control
10 mM
0.1 mM
1X
1:100 Diluted
1X
1:100 Diluted
- +TNF - +TNF+TNF - +TNF - +TNF - +TNF
NFkB/DNA EMSA Product
NFkB/DNA EMSA Unknown Seconday Product

FIG.10C

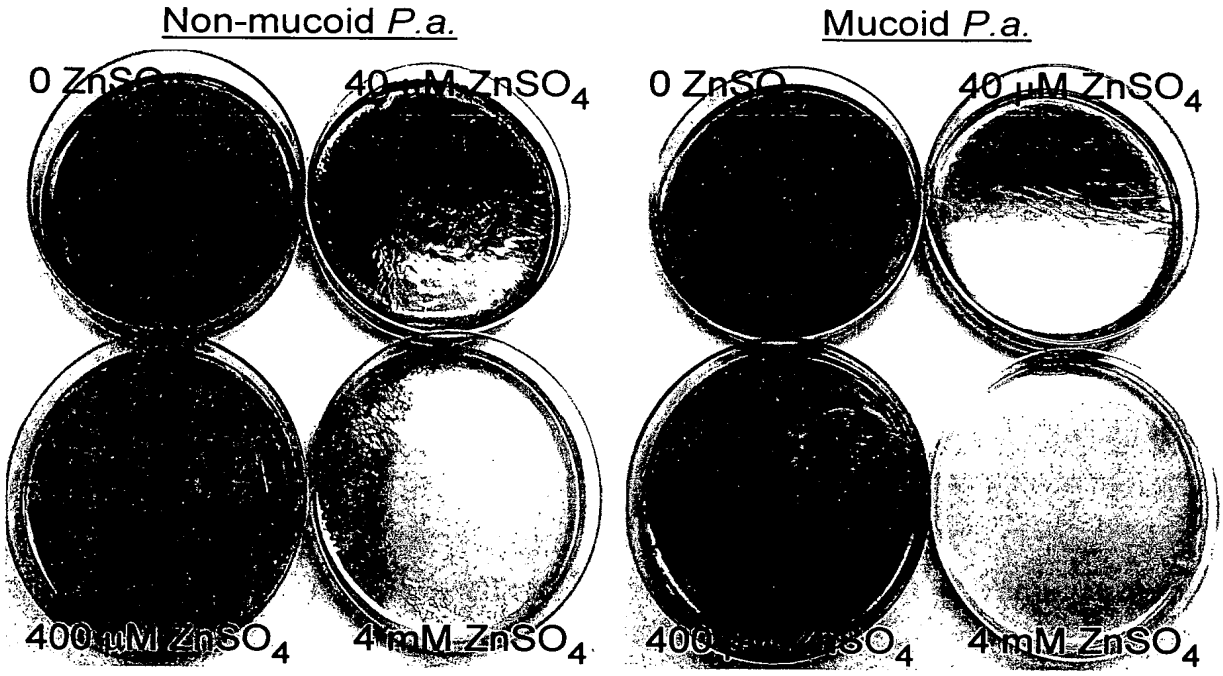
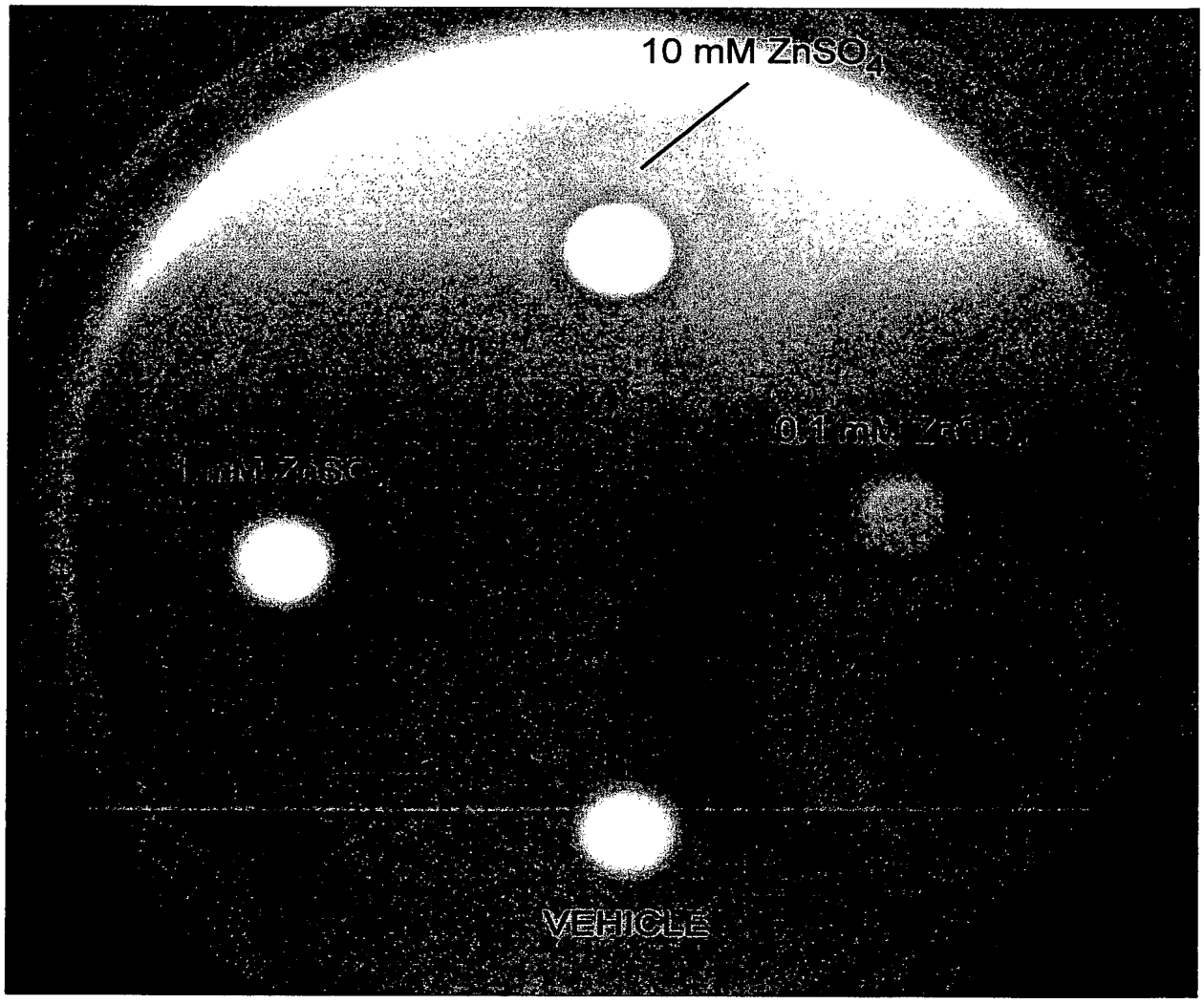


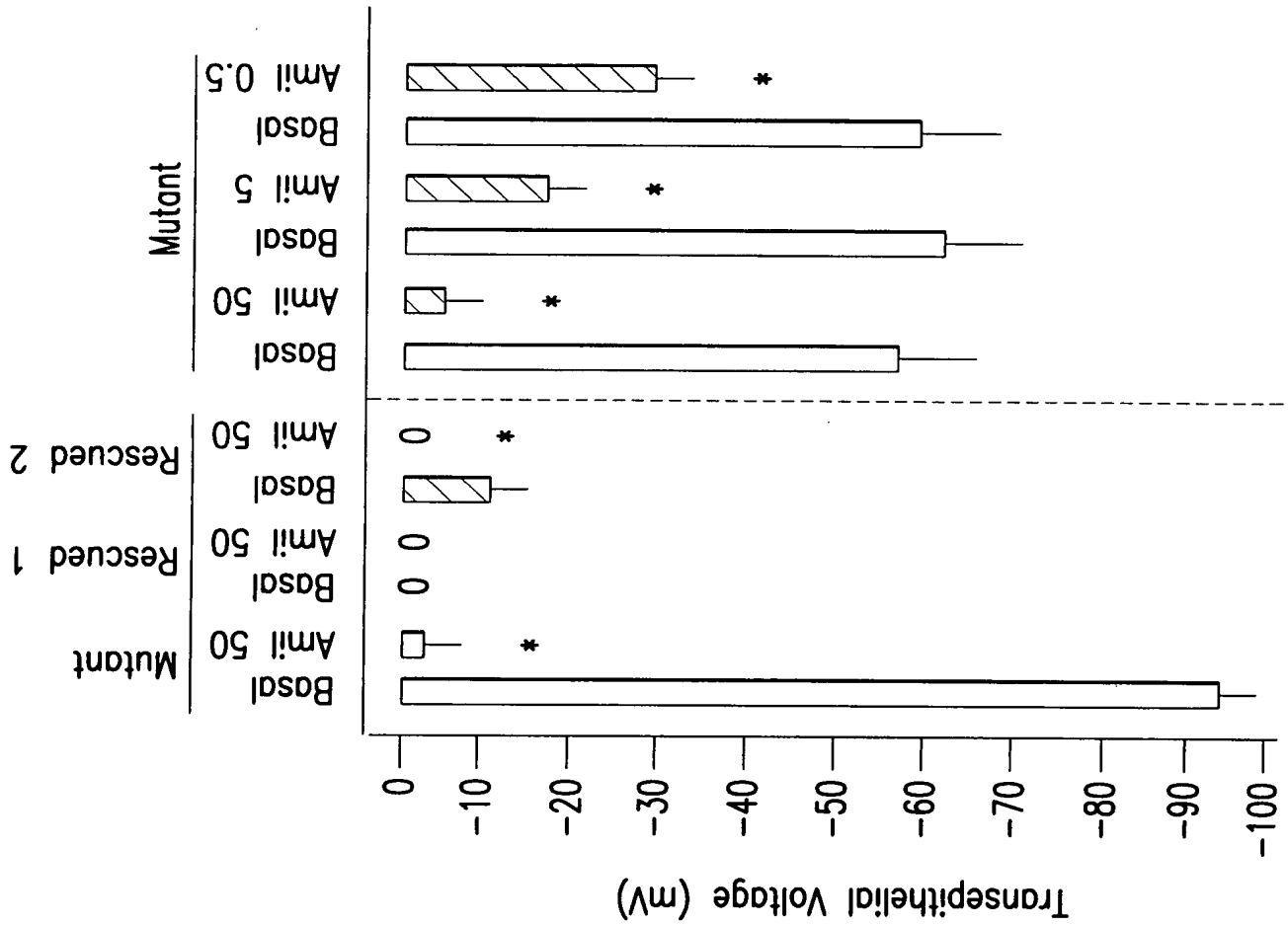
FIG. 11A

FIG. 11B



E. coli.

FIG. 11C


Transepithelial Voltage (mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Mutant
Basal
Amil 0.5
Basal
Amil 5
Basal
Amil 50
Basal
Rescued 2
Amil 50
Basal
Rescued 1
Amil 50
Basal
Mutant
Amil 50
Basal
0
0
0
*
*
*
*
*

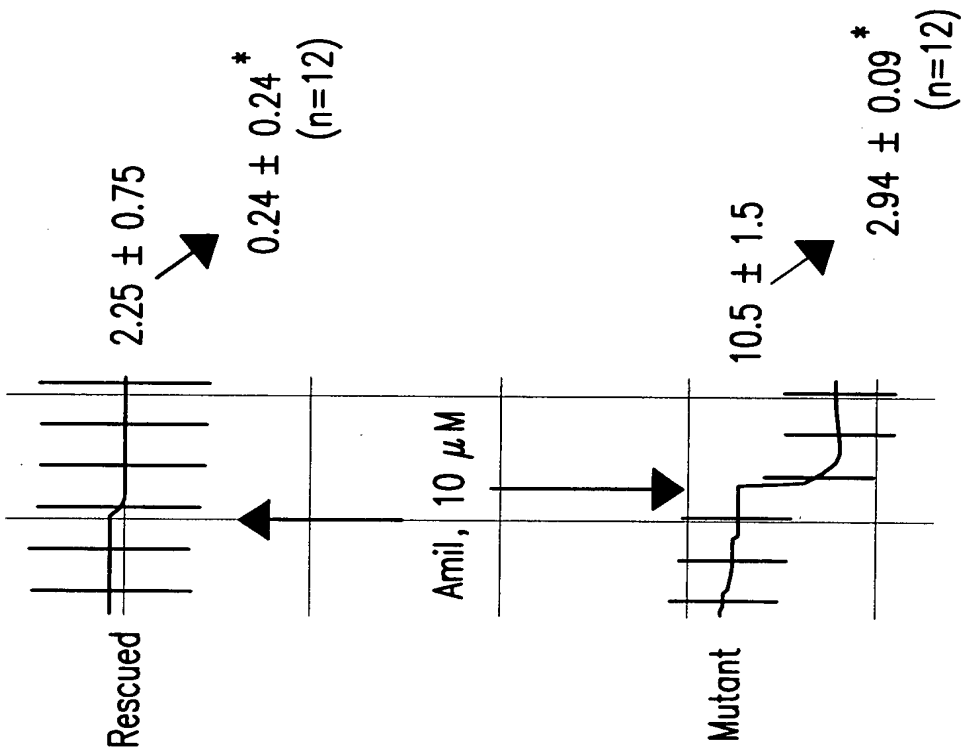
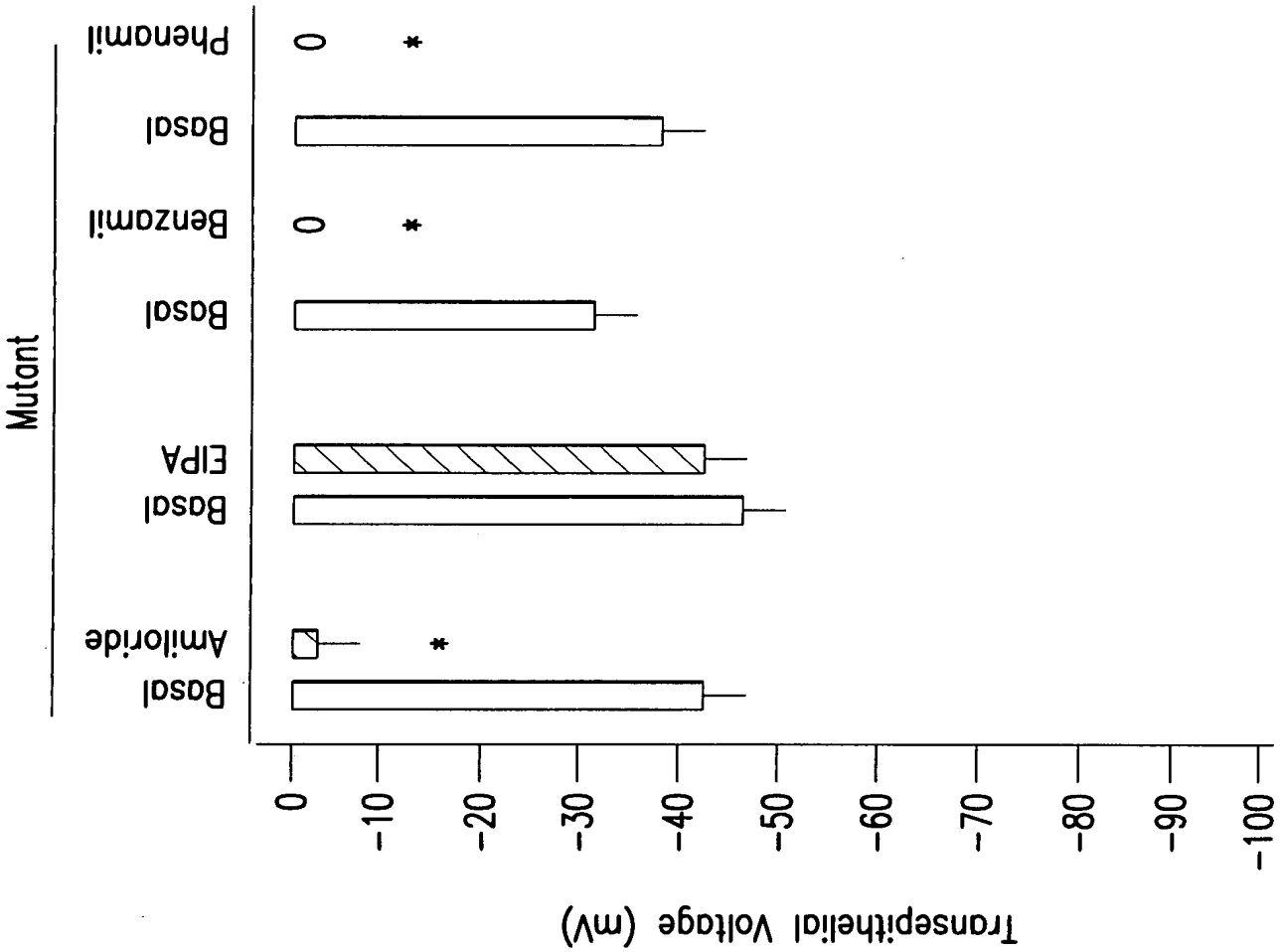

Rescued
Amil, 10 μM
Mutant
2.25 ± 0.75
0.24 ± 0.24*
(n=12)
10.5 ± 1.5
2.94 ± 0.09*
(n=12)

FIG. 12A


Mutant
Basal
Amiloride
Basal
EIPA
Basal
Benzamil
Basal
Phenamil
*
*
*
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Transepithelial Voltage (mV)

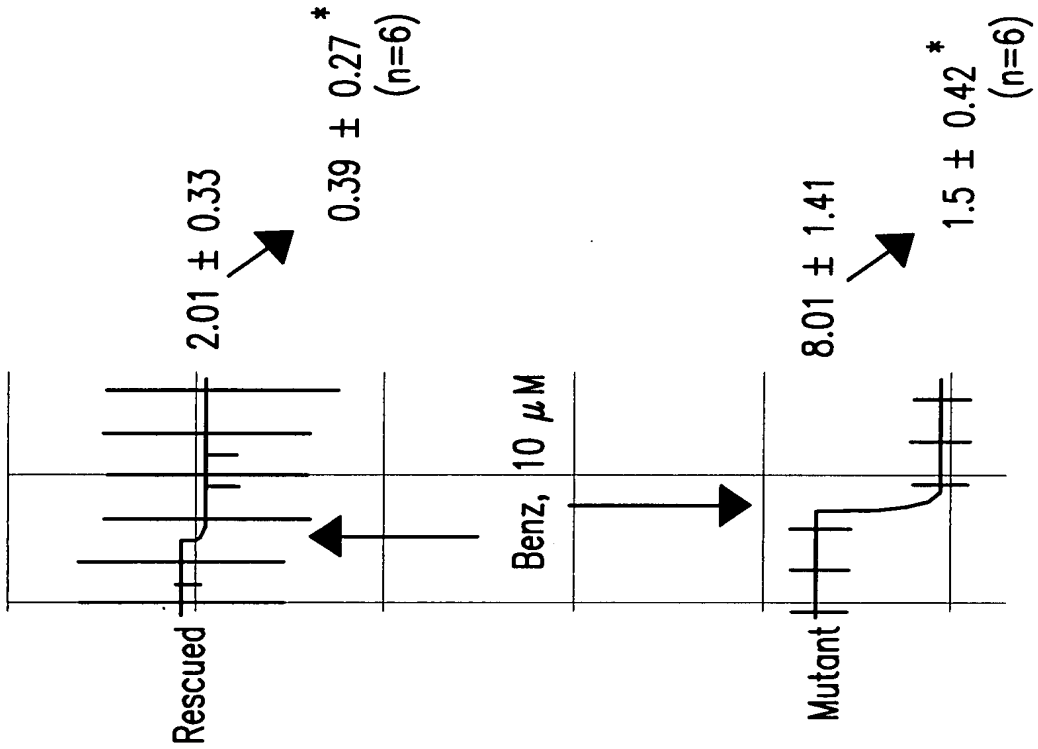

Rescued
Benz, 10 μM
Mutant
2.01 ± 0.33
0.39 ± 0.27 * (n=6)
8.01 ± 1.41
1.5 ± 0.42 * (n=6)

FIG. 12B

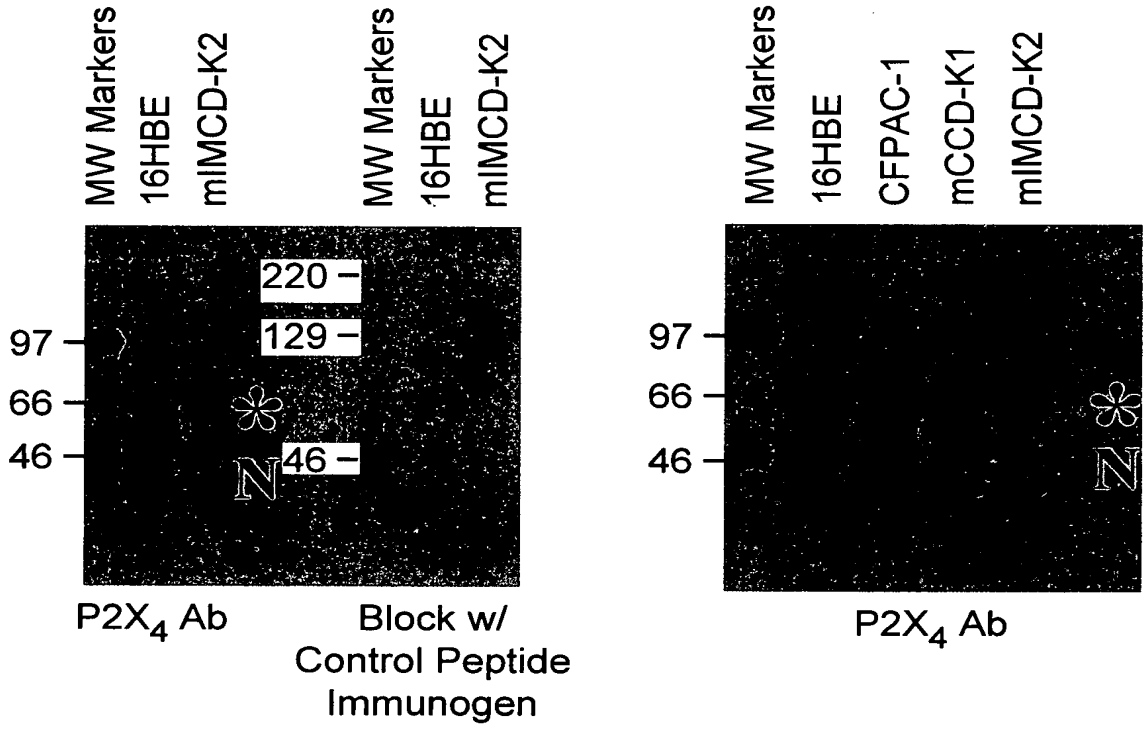

MW Markers
16HBE
mIMCD-K2
MW Markers
16HBE
mIMCD-K2
MW Markers
16HBE
CFPAC-1
mCCD-K1
mIMCD-K2
220
129
46
97
66
46
97
66
46
N
N
P2X4 Ab
Block w/ Control Peptide Immunogen
P2X4 Ab

FIG. 13A

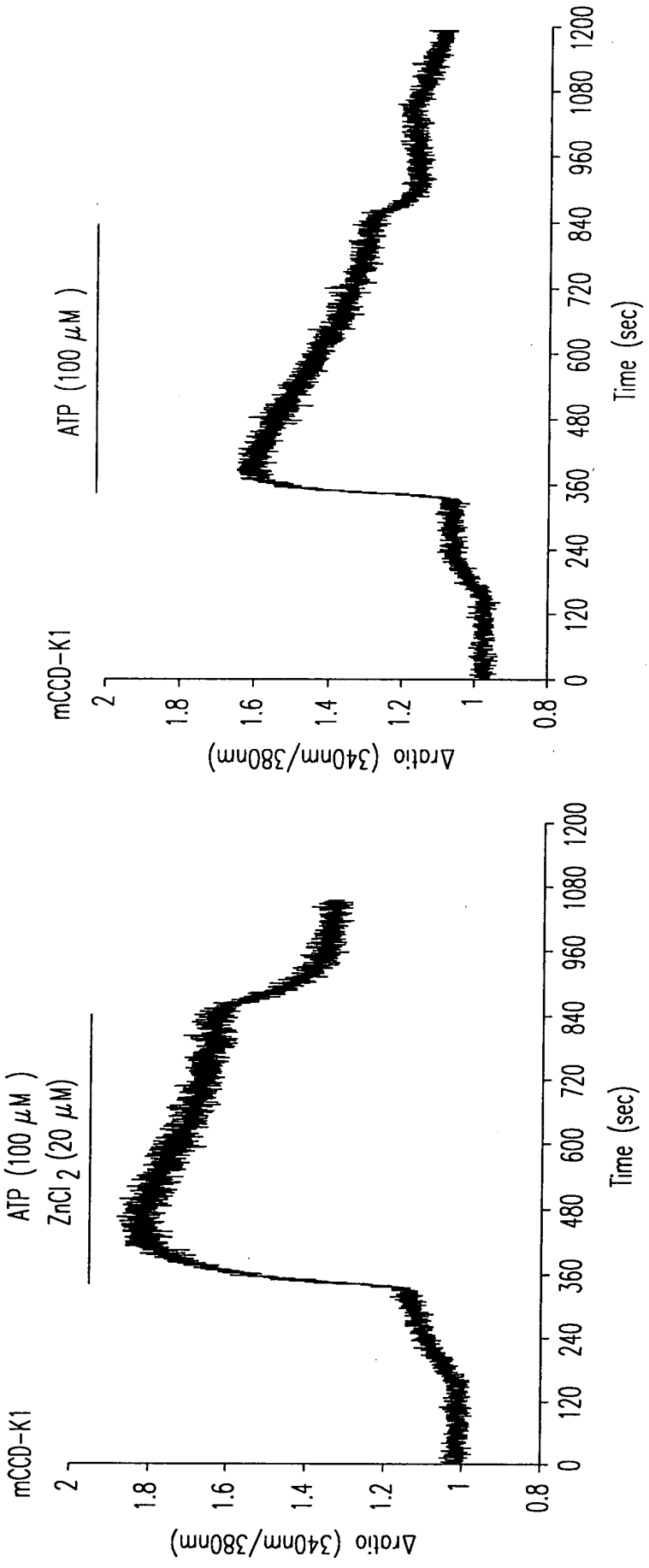

mCCD-K1
ATP (100 μM)
ZnCl 2 (20 μM)
Δratio (340nm/380nm)
Time (sec)
mCCD-K1
ATP (100 μM)
Δratio (340nm/380nm)
Time (sec)

FIG.13B-1

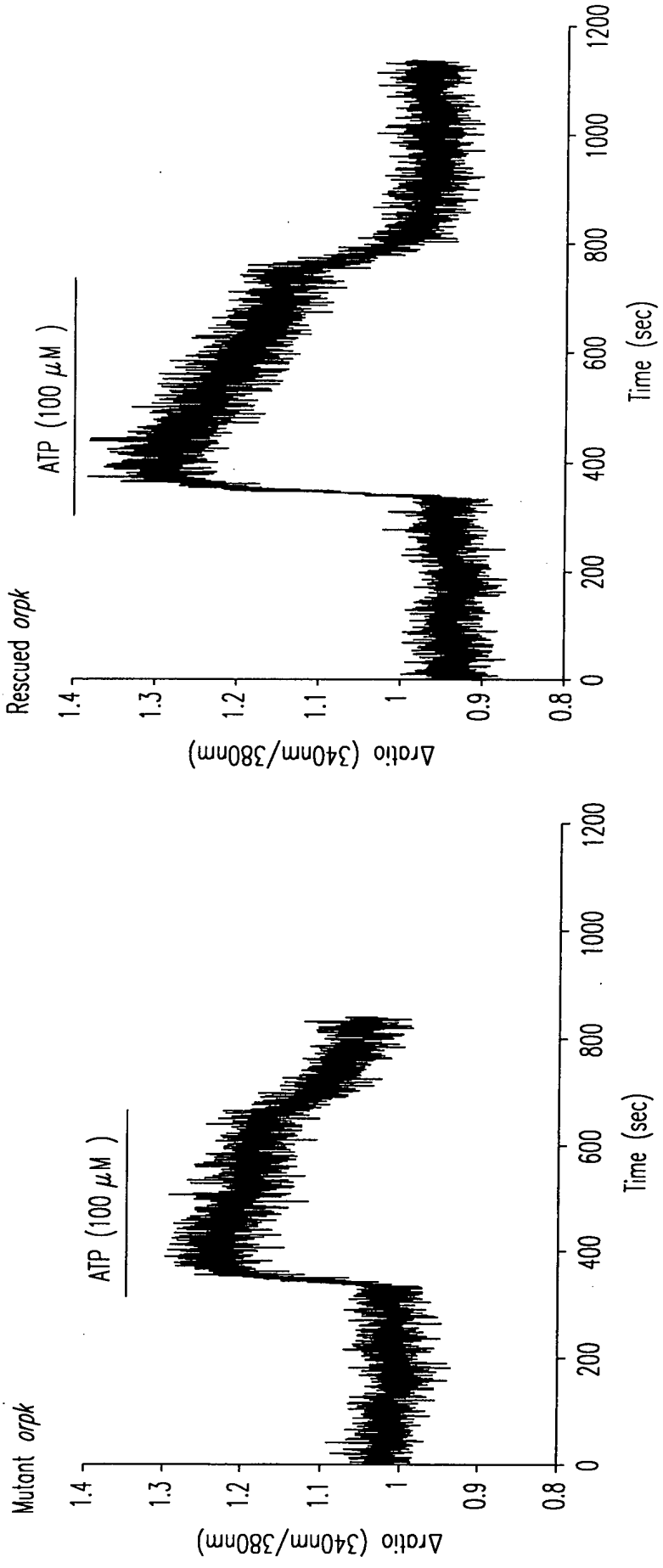

Rescued orpk
ATP (100 μM)
Δratio (340nm/380nm)
Time (sec)
Mutant orpk
ATP (100 μM)
Δratio (340nm/380nm)
Time (sec)

FIG.13B-2

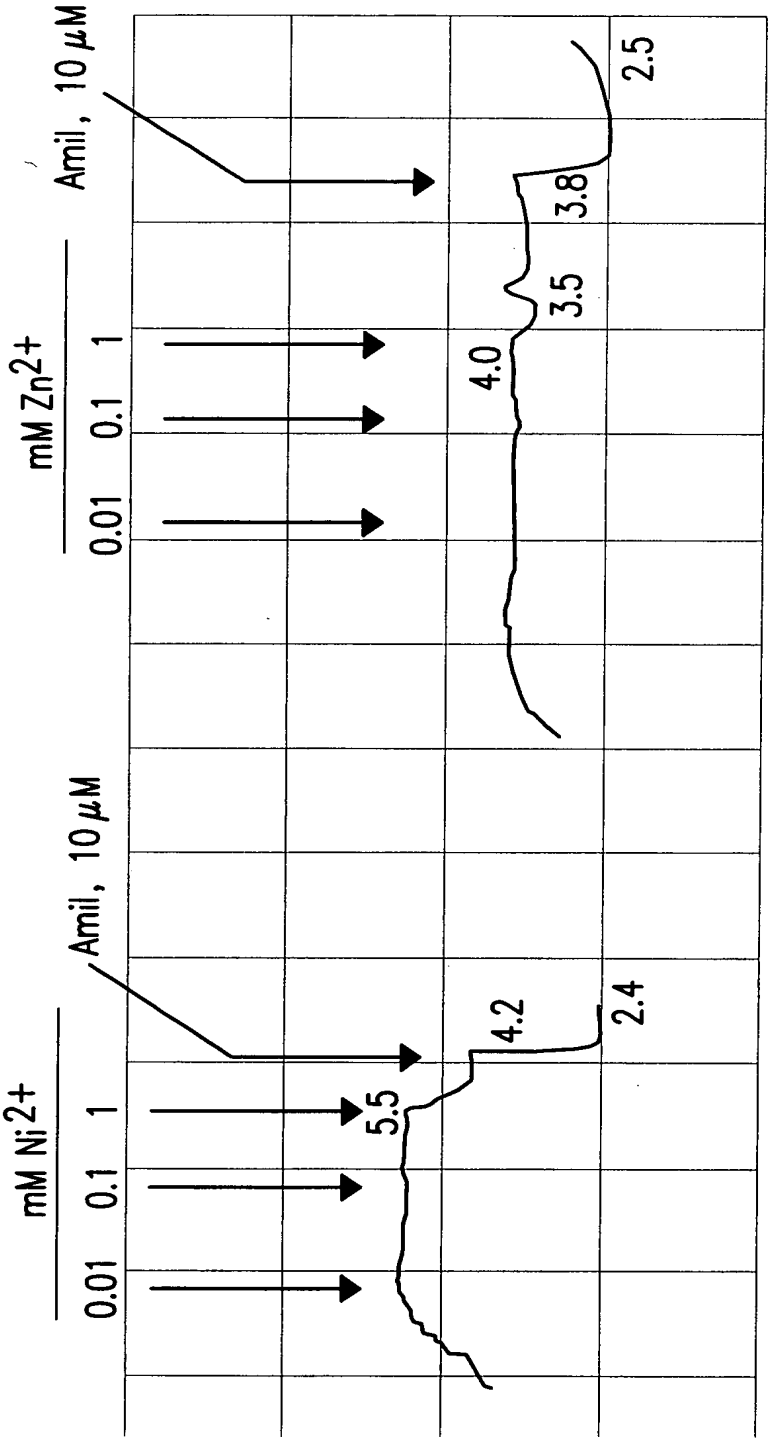

mM Ni2+
0.01
0.1
1
Amil, 10 μM
5.5
4.2
2.4
mM Zn2+
0.01
0.1
1
Amil, 10 μM
4.0
3.5
3.8
2.5

FIG. 14A

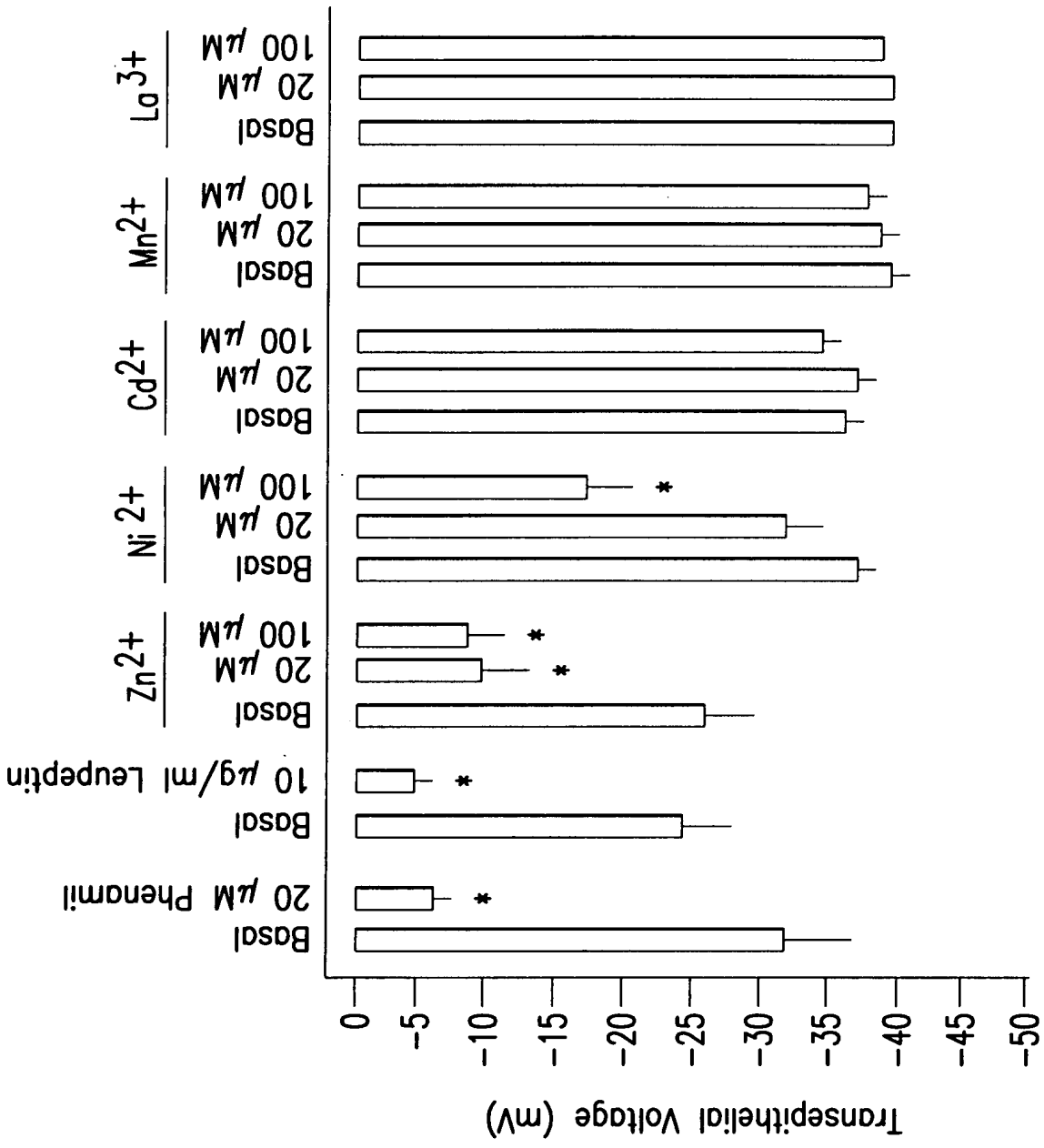

Transepithelial Voltage (mV)
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
-50
Basal
20 μM Phenamil
Basal
10 μg/ml Leupeptin
Basal
20 μM
100 μM
Zn2+
Basal
20 μM
100 μM
Ni2+
Basal
20 μM
100 μM
Cd2+
Basal
20 μM
100 μM
Mn2+
Basal
20 μM
100 μM
La3+

FIG. 14B

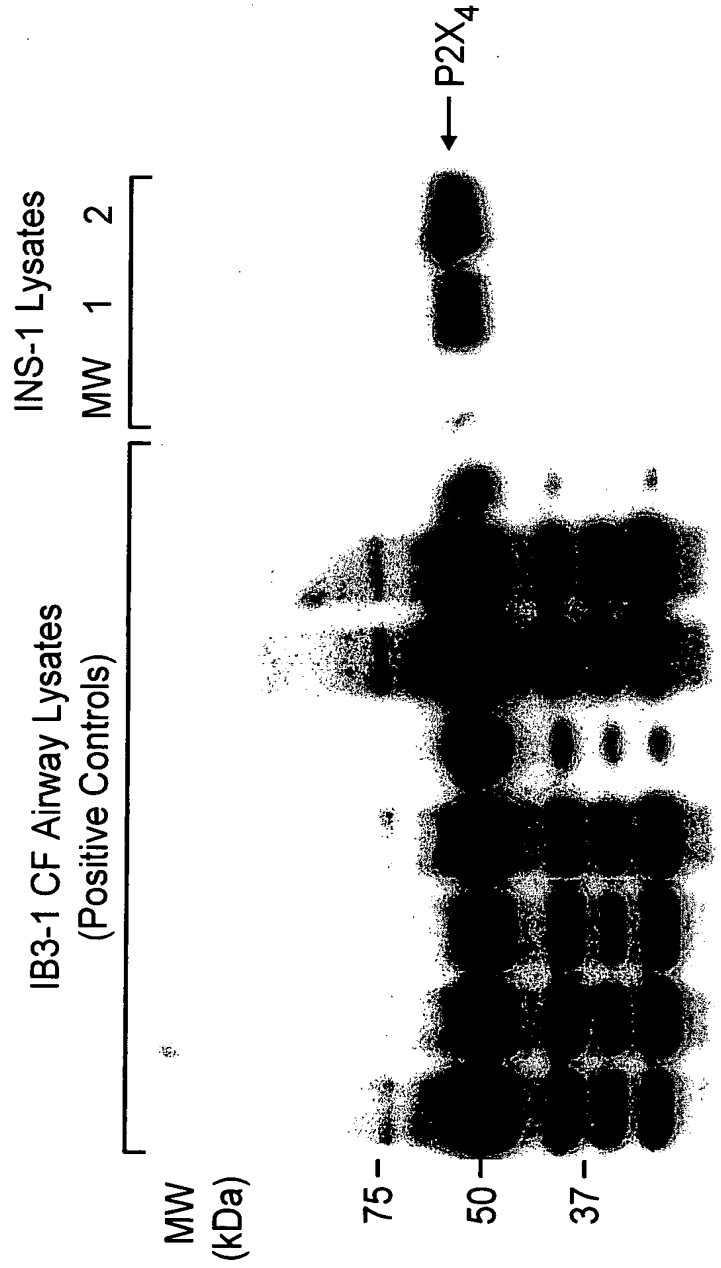

INS-1 Lysates
MW 1 2
IB3-1 CF Airway Lysates
(Positive Controls)
P2X4
MW
(kDa)
75
50
37

FIG.15A

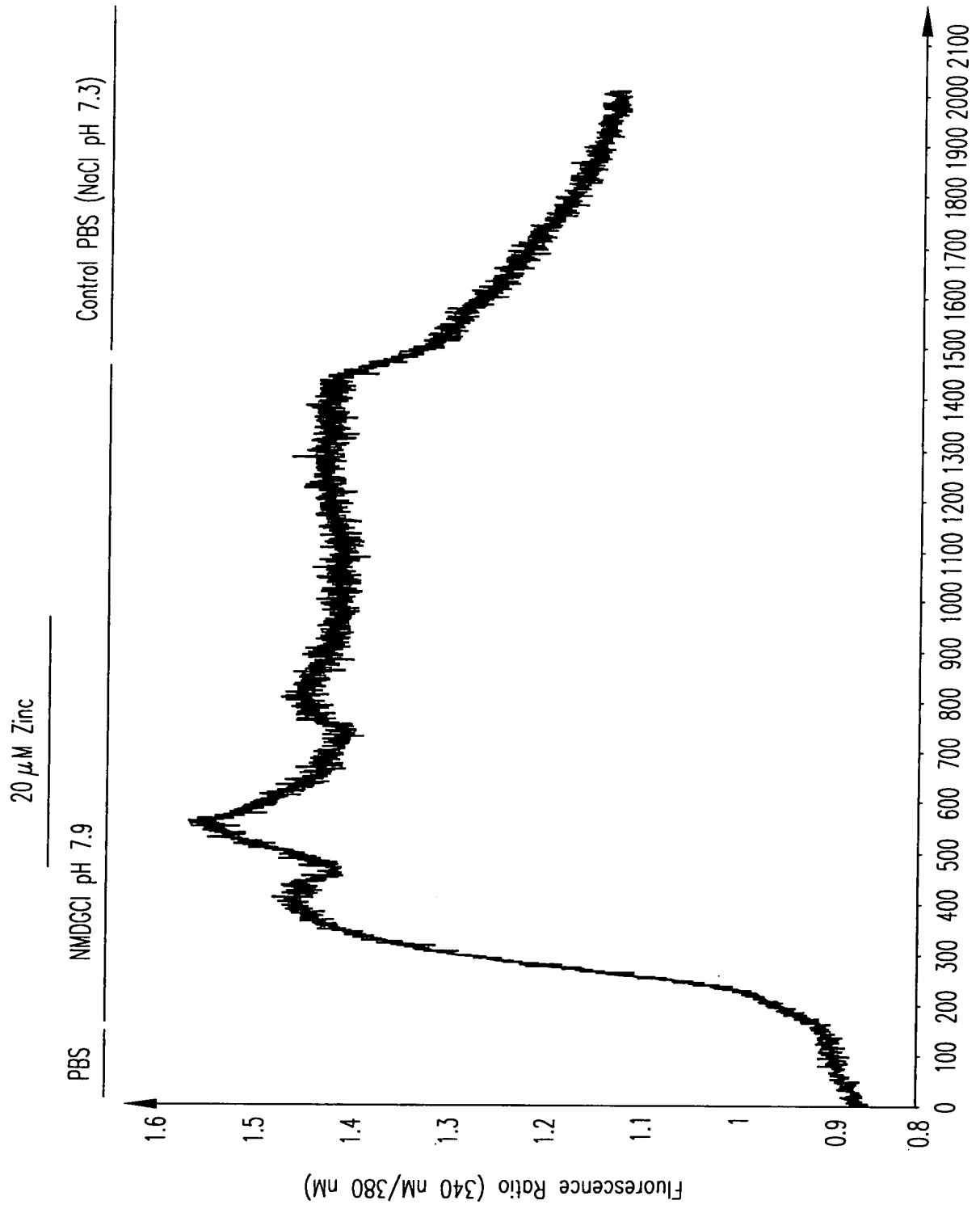

PBS
NMDGCl pH 7.9
20 μM Zinc
Control PBS (NaCl pH 7.3)
Fluorescence Ratio (340 nM/380 nM)
Time (seconds)
0.8
0.9
1
1.1
1.2
1.3
1.4
1.5
1.6
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100

FIG.15B

Modified Saline** (pH 7.3)			Modified Saline (pH 7.3) + 15 mM Glucose		
Time	Absorbance	[Insulin]	Time	Absorbance	[Insulin]
15"	0.682 ± 0.03	~3.0 ng/ml	15"	1.070 ± 0.05	~5.0 ng/ml
15'	0.765 ± 0.04	3.25	15'	0.957 ± 0.07	4.5
30'	0.794 ± 0.06	3.5	30'	1.204 ± 0.10	5.5
60'	1.794 ± 0.09	9.0	60'	2.065 ± 0.05	11.0
120'	1.137 ± 0.05	5.0	120'	1.105 ± 0.18	5.0

*Generous gift of Dr. Chris Newgard at Duke.

**Modified saline is 0 Na (substituted fully by NMDG), 0 Mg, and 3 mM Ca.

Standard Curve

Absorbance	[Insulin]
0.248	0.0
0.226	0.2 ng/ml
0.280	0.5 ng/ml
0.377	1.0 ng/ml
0.559	2.0 ng/ml
1.10	5.0 ng/ml
1.91	10.0 ng/ml
~3.0	~20 ng.ml

FIG.16A

Inventor: Schwiebert et al.
Title: Methods and Compositions for P2X Receptor Calcium Entry Channels and Other Calcium Entry Mechanisms
Docket No.: 21085.0044U3
REPLACEMENT SHEET
Sheet 36 of 51

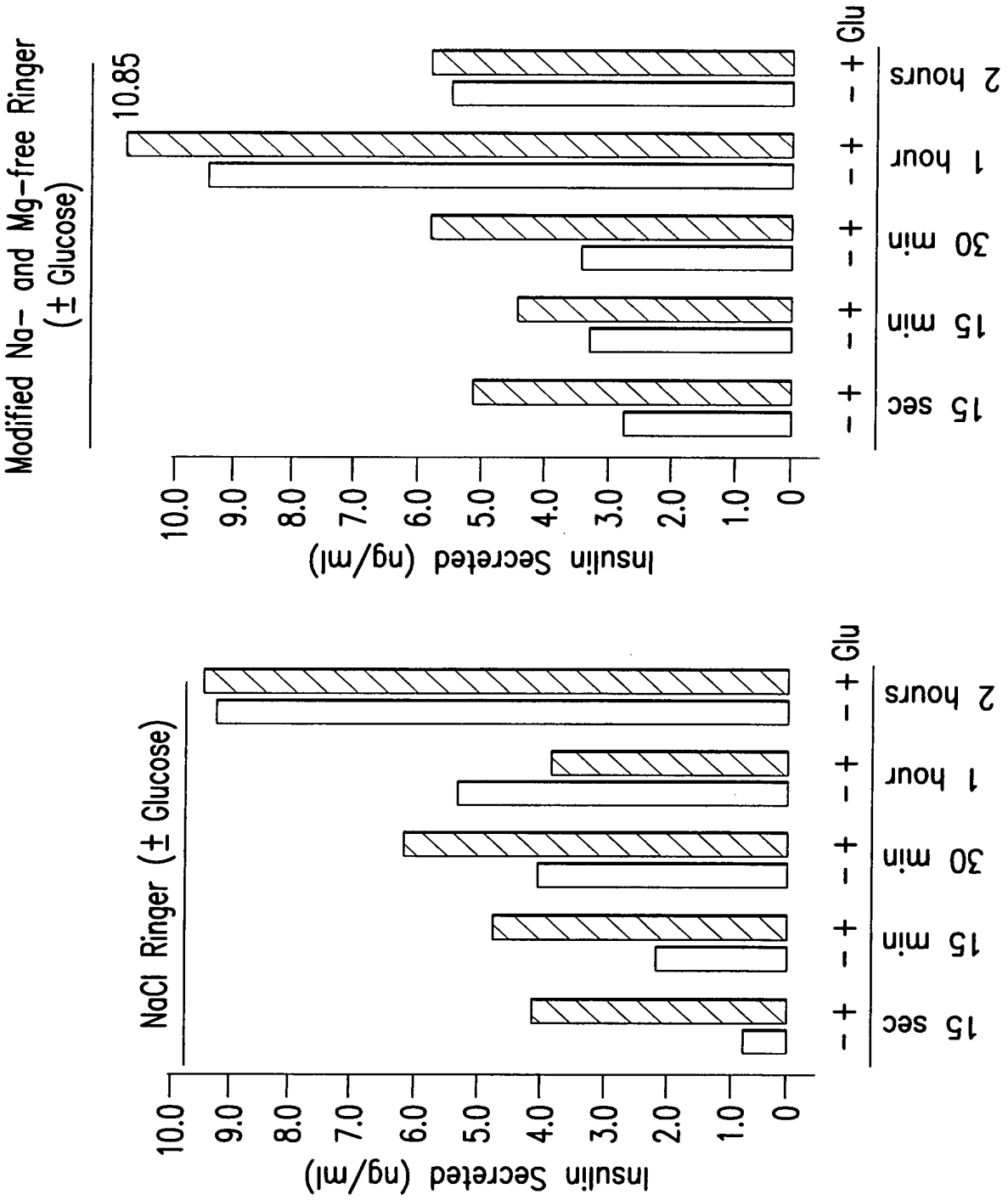

Modified Na- and Mg-free Ringer (± Glucose)
10.85
NaCl Ringer (± Glucose)
Insulin Secreted (ng/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours
- Glu
+ Glu

FIG.16B

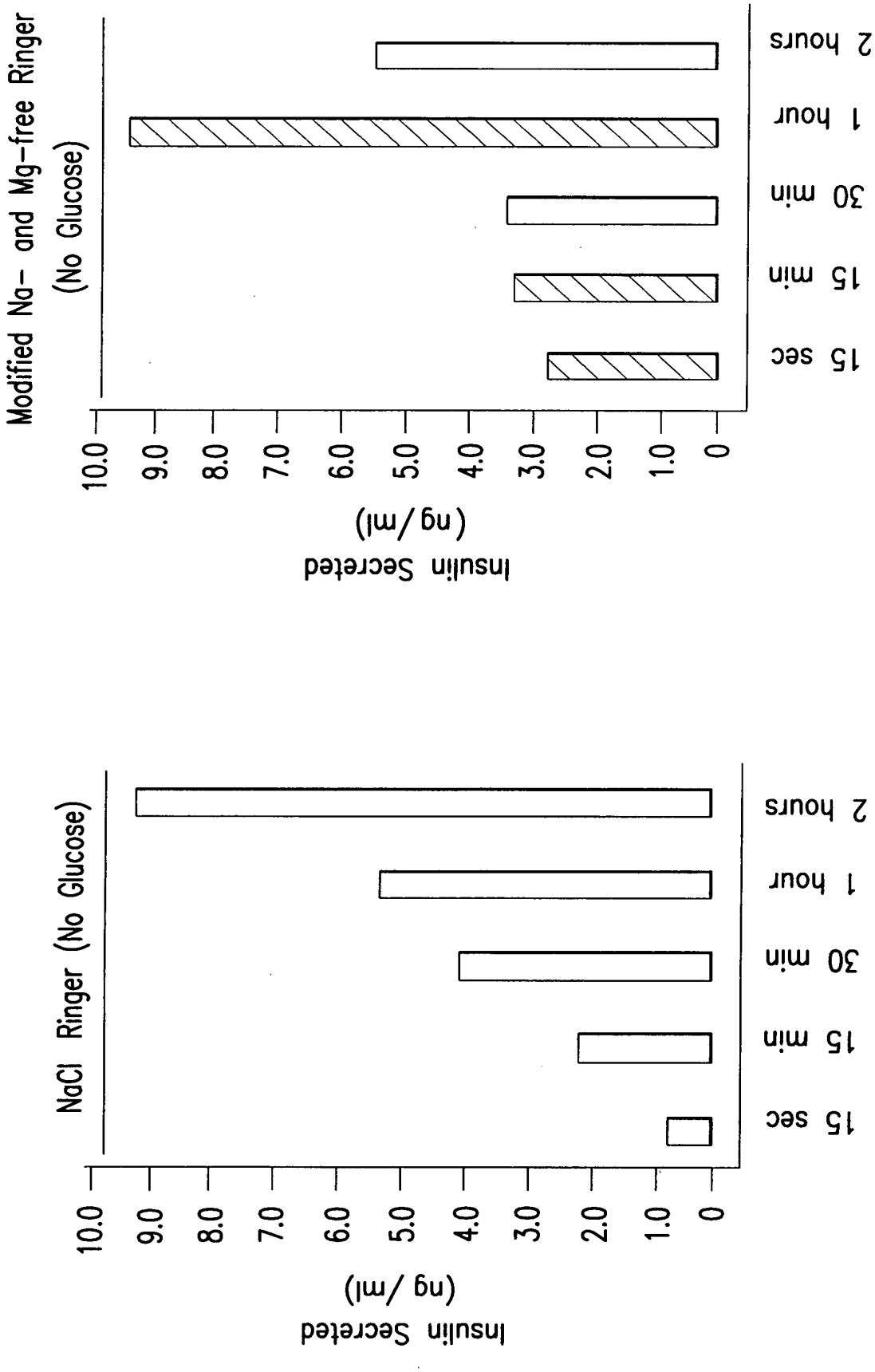
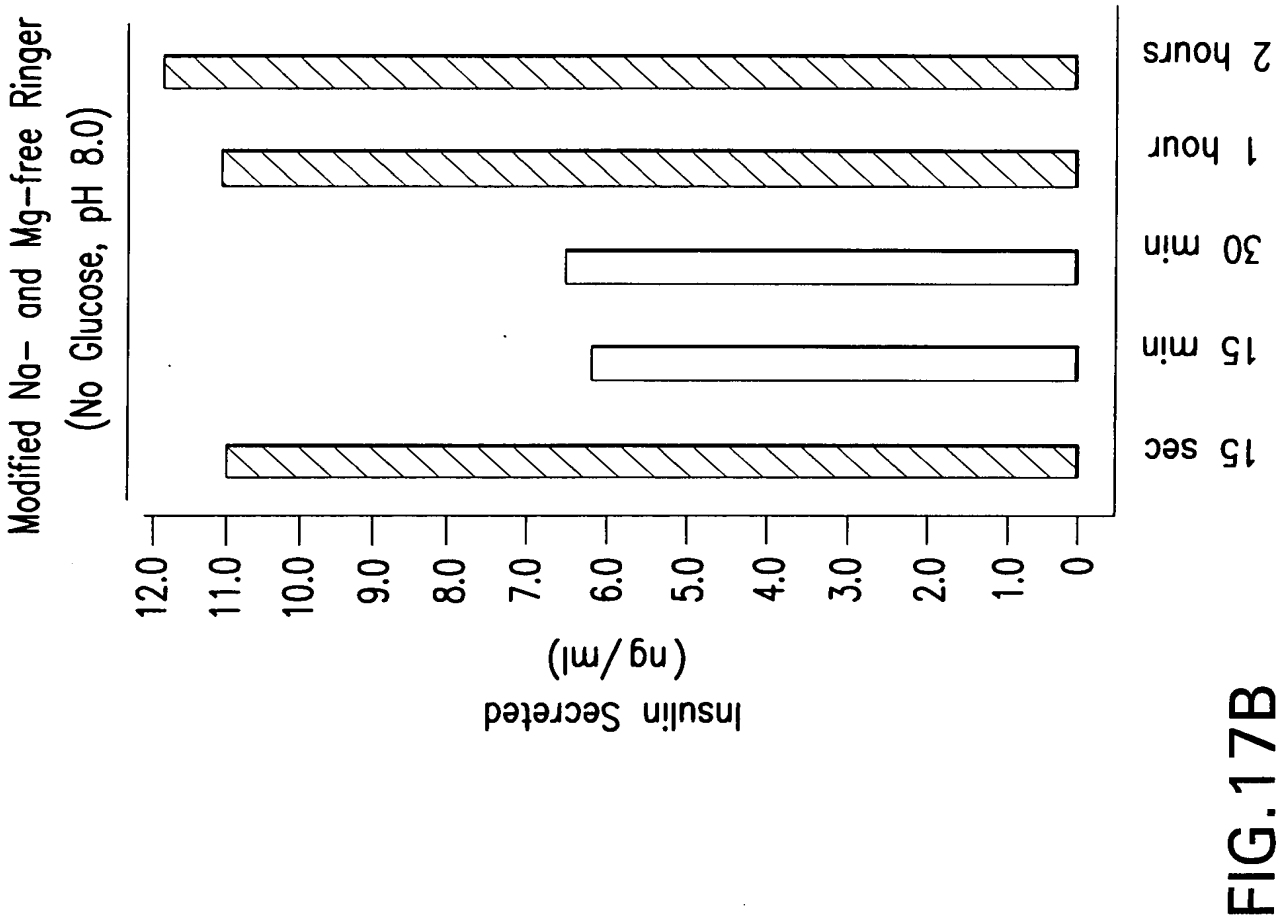

Modified Na- and Mg-free Ringer
(No Glucose)
Insulin Secreted
(ng/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours
NaCl Ringer (No Glucose)
Insulin Secreted
(ng/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours

FIG.17A


Modified Na- and Mg-free Ringer
(No Glucose, pH 8.0)
Insulin Secreted
(ng /ml)
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours

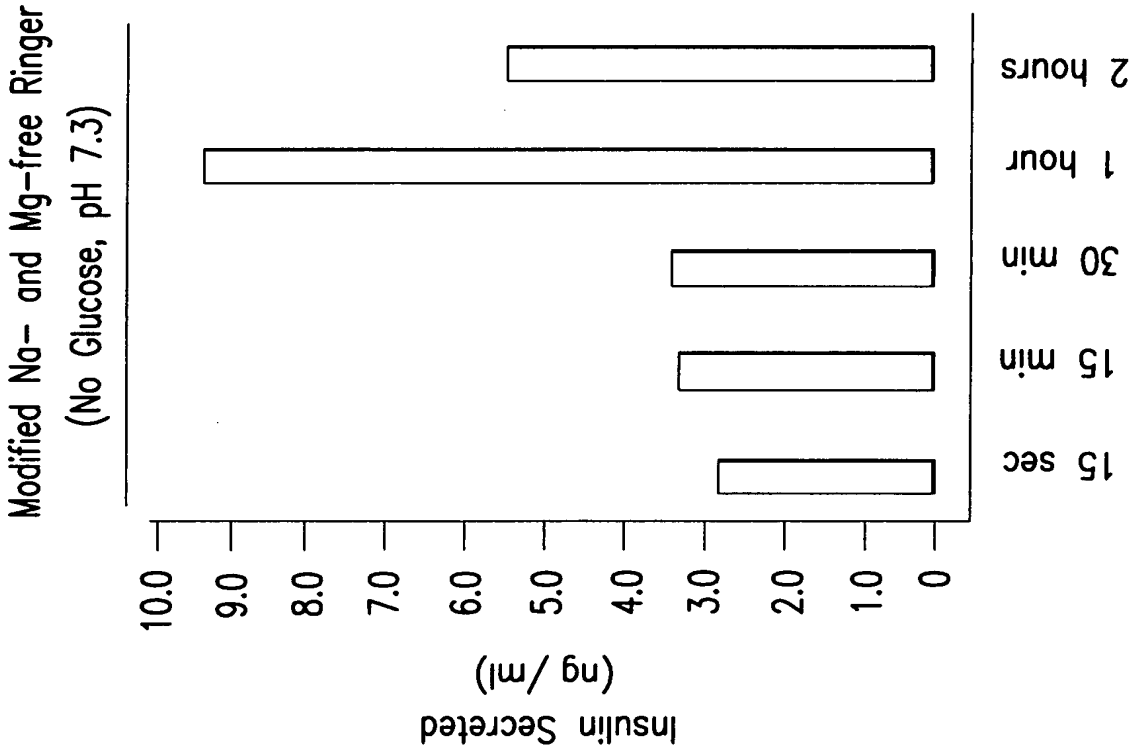

Modified Na- and Mg-free Ringer
(No Glucose, pH 7.3)
Insulin Secreted
(ng /ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours

FIG.17B

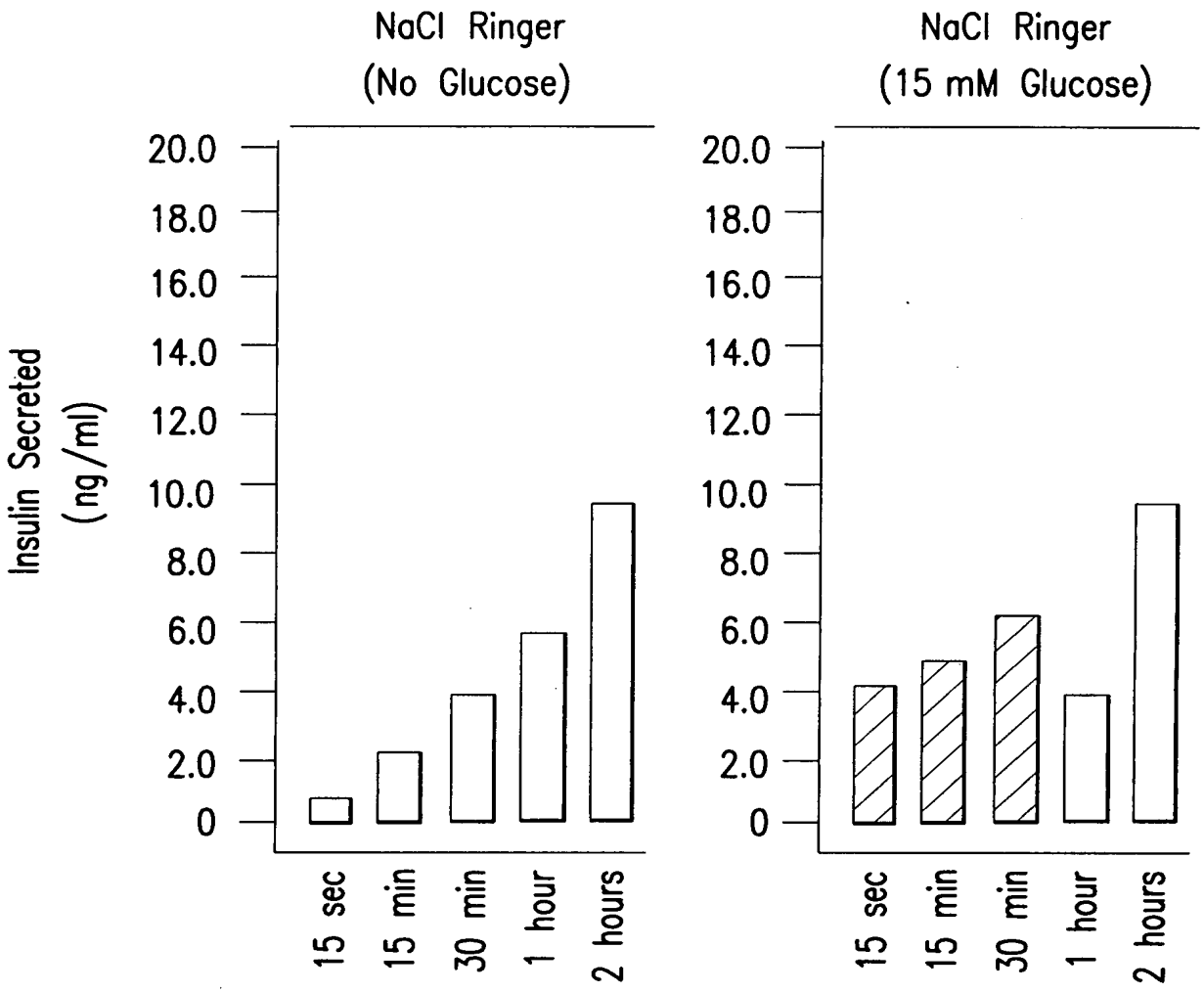

NaCl Ringer
(No Glucose)
NaCl Ringer
(15 mM Glucose)
Insulin Secreted
(ng/ml)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
15 sec
15 min
30 min
1 hour
2 hours

FIG.18A

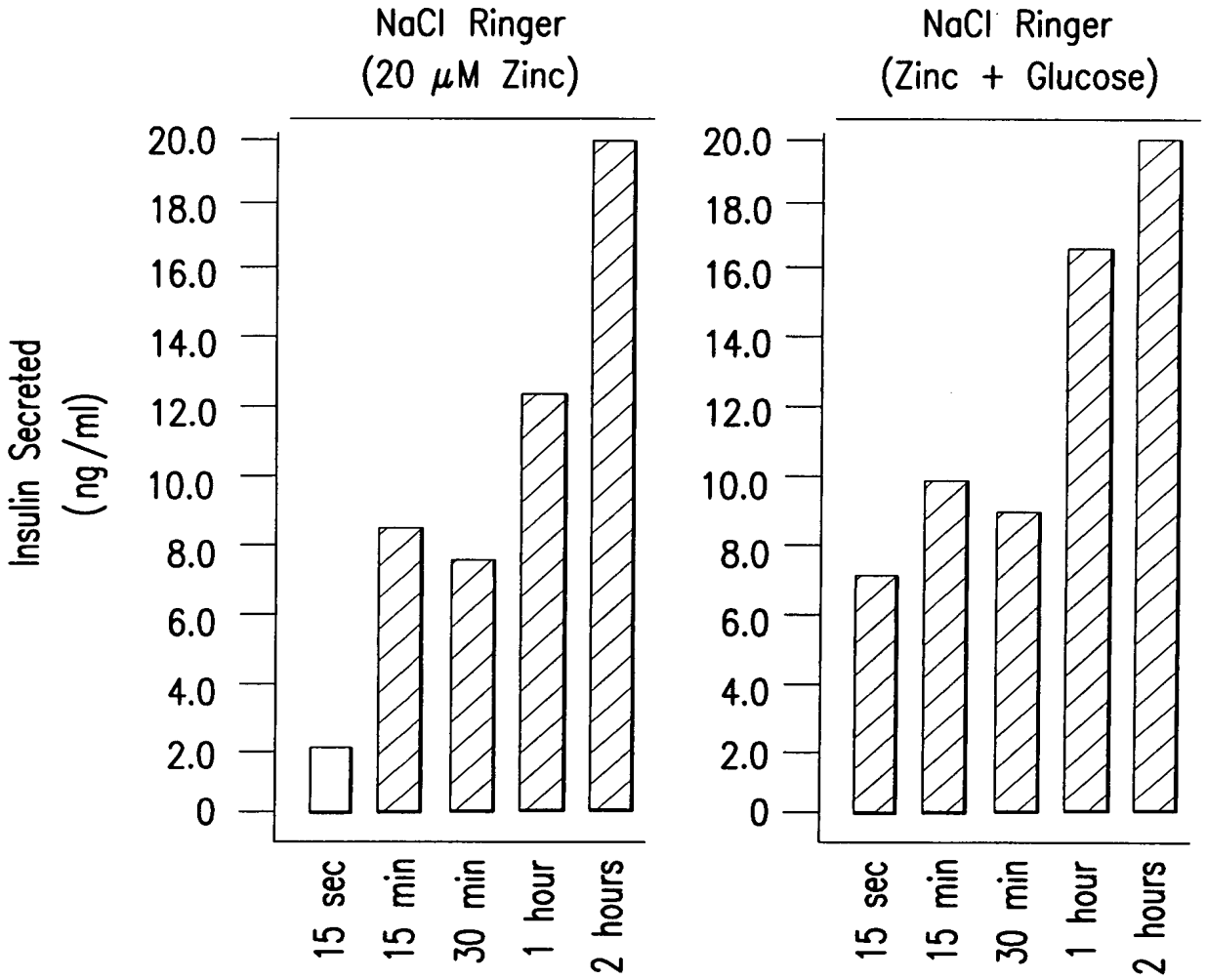

NaCl Ringer
(20 μM Zinc)
NaCl Ringer
(Zinc + Glucose)
Insulin Secreted
(ng/ml)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
15 sec
15 min
30 min
1 hour
2 hours

FIG.18B

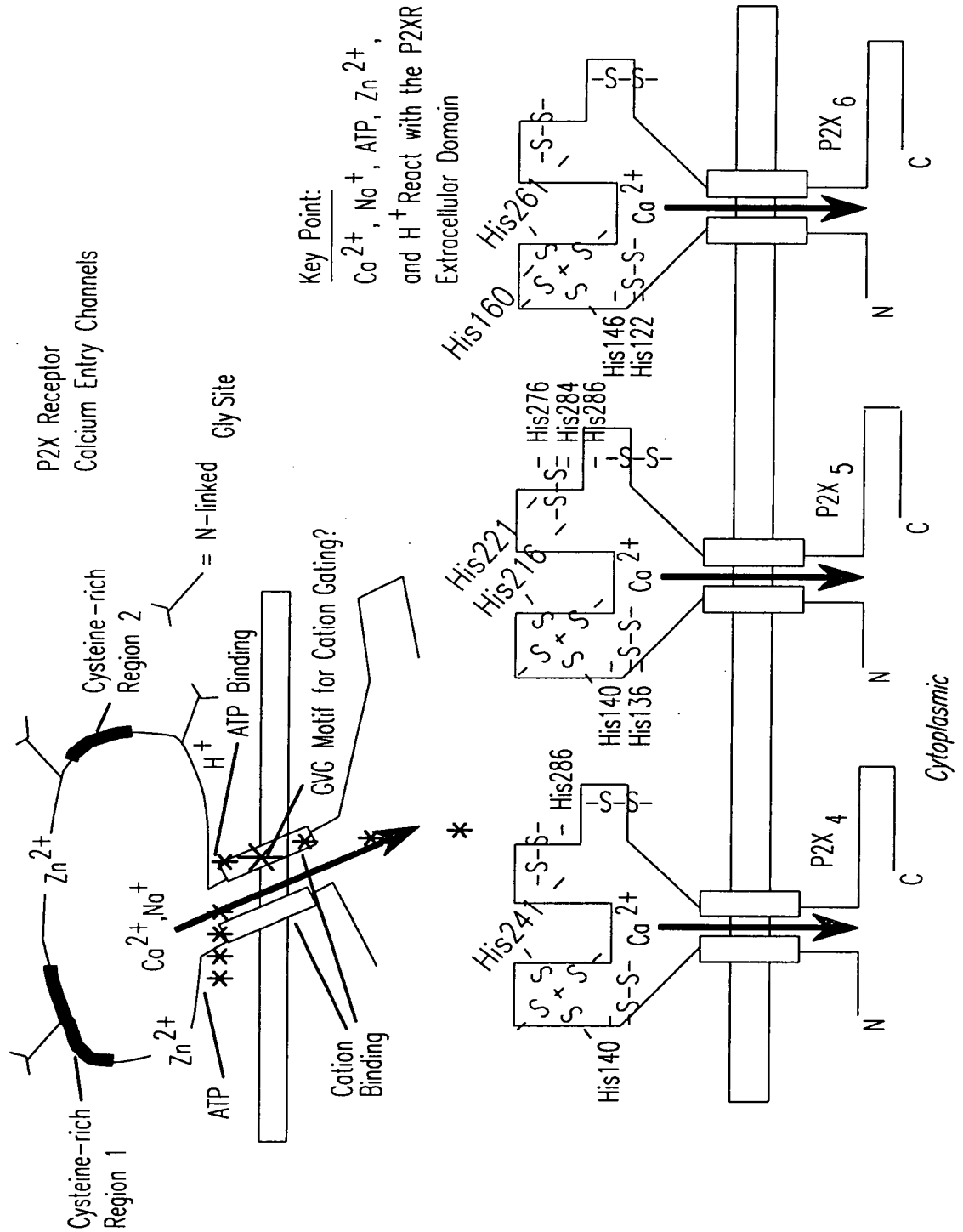

P2X Receptor
Calcium Entry Channels
Key Point:
Ca^{2+}, Na^+, ATP, Zn^{2+}, and H^+ React with the P2XR Extracellular Domain
= N-linked Gly Site
Cysteine-rich Region 2
Cysteine-rich Region 1
Zn^{2+}
H^+
ATP Binding
GVG Motif for Cation Gating?
Ca^{2+},Na^+
Zn^{2+}
ATP
Cation Binding
His261
His160
His146
His122
His276
His284
His286
His221
His216
His140
His136
His286
His241
His140
P2X 6
P2X 5
P2X 4
N
C
Cytoplasmic

FIG.19A

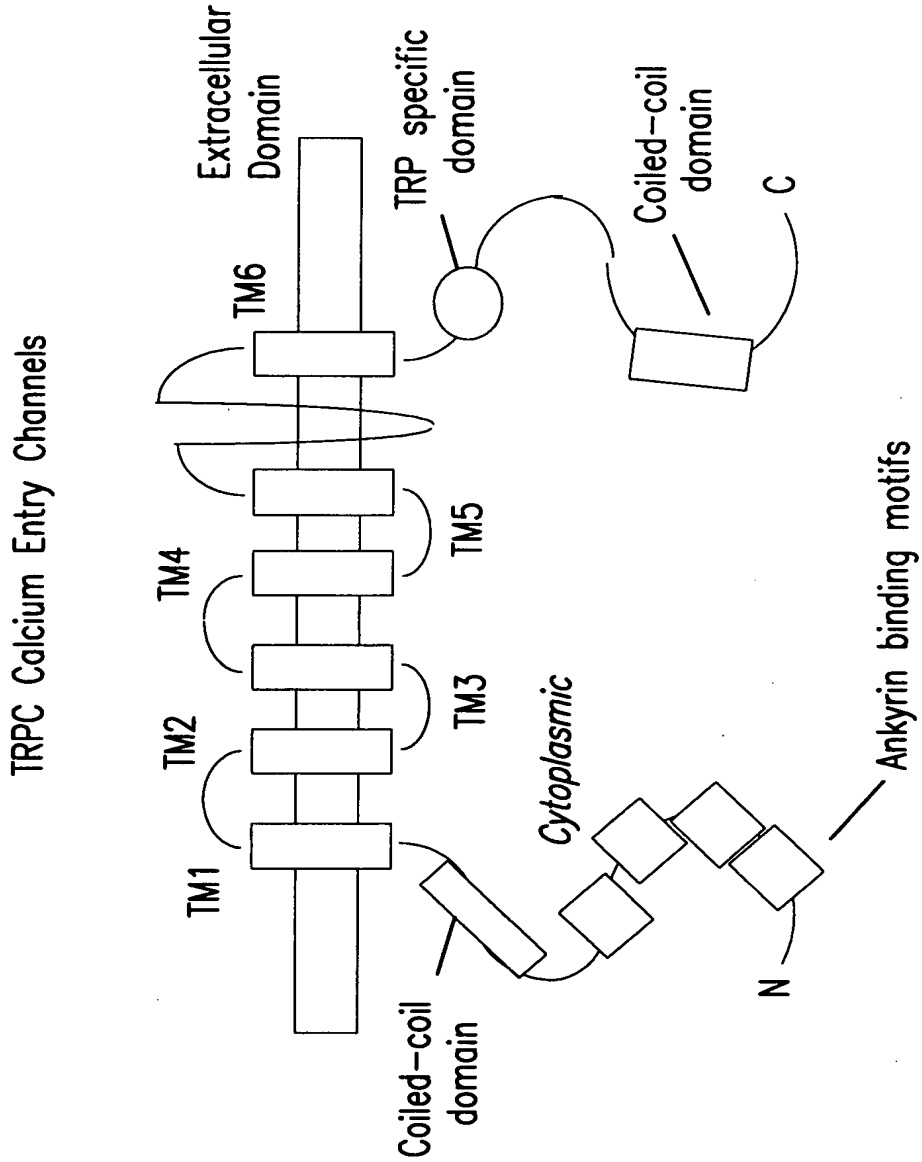
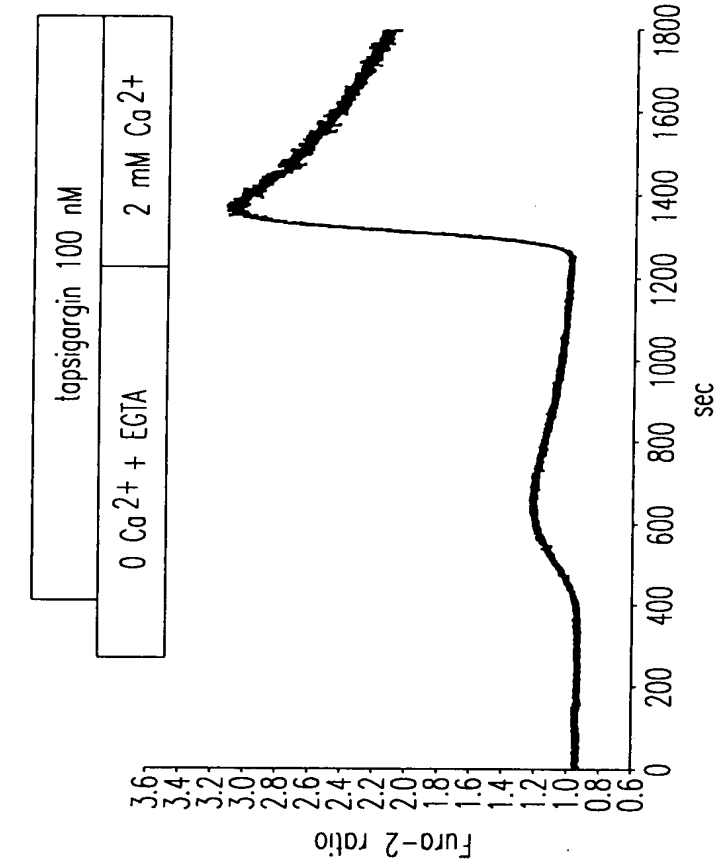

TRPC Calcium Entry Channels
Extracellular Domain
TRP specific domain
Coiled-coil domain
C
TM6
TM5
TM4
TM3
TM2
TM1
Cytoplasmic
Ankyrin binding motifs
N
Coiled-coil domain

FIG.19B


thapsigargin 100 nM
0 Ca2+ + EGTA
2 mM Ca2+
Fura-2 ratio
sec
0
200
400
600
800
1000
1200
1400
1600
1800
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6

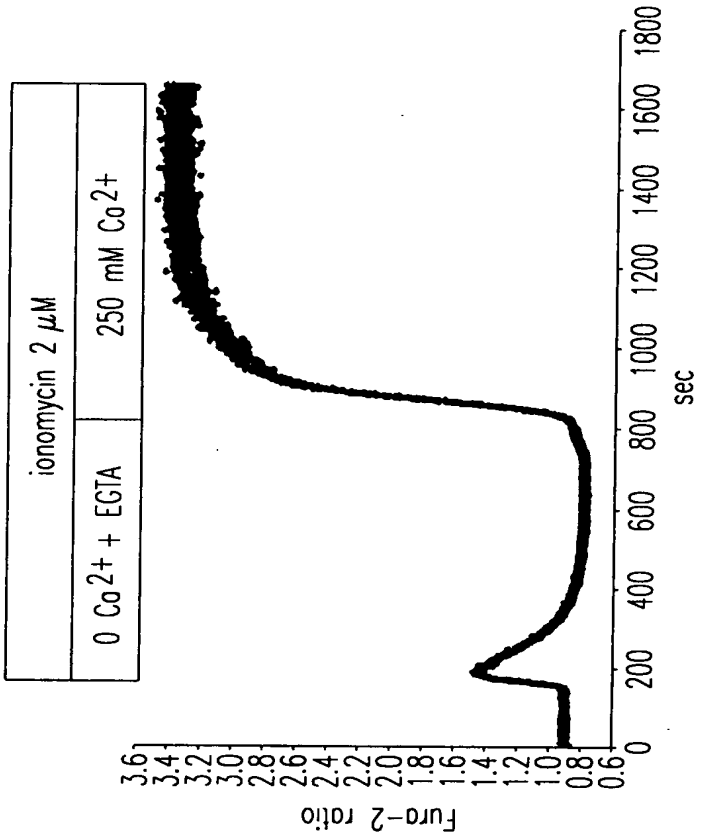
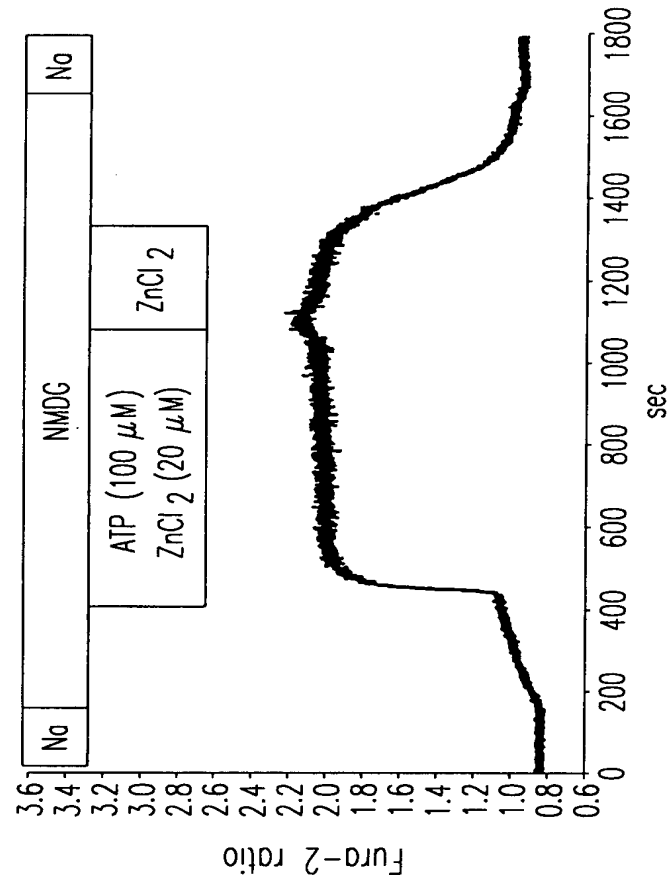

ionomycin 2 μM
0 Ca2+ + EGTA
250 mM Ca2+
Fura-2 ratio
sec
0
200
400
600
800
1000
1200
1400
1600
1800
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6

FIG. 19C-1


Fura-2 ratio
sec
Na
NMDG
Na
ATP (100 μM)
ZnCl 2 (20 μM)
ZnCl 2

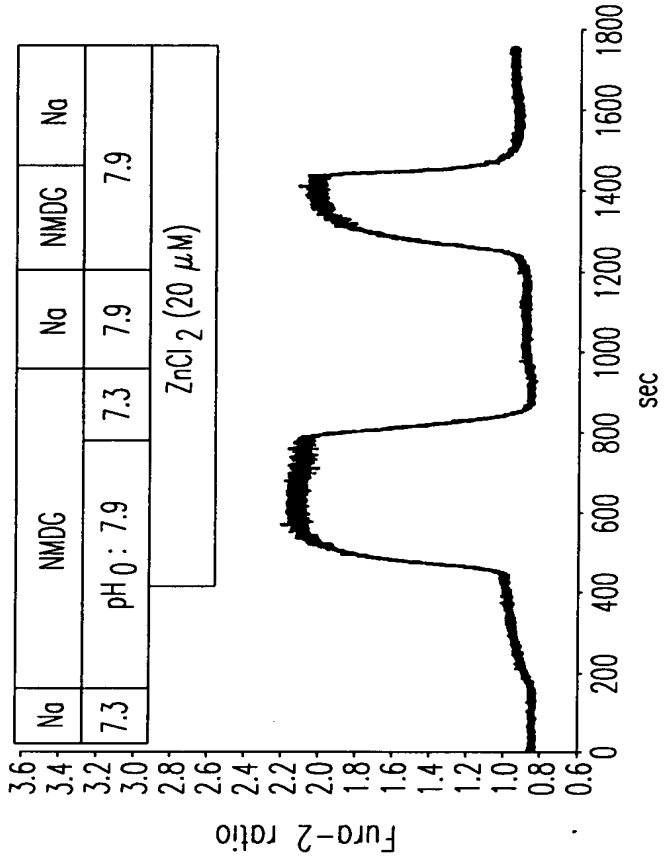

Fura-2 ratio
sec
Na
NMDG
Na
NMDG
Na
7.3
pH 0 : 7.9
7.3
7.9
7.9
ZnCl 2 (20 μM)

FIG.19C-2

Designation	Mode of Stimulation	Epithelial Polarity
Store-operated Ca^{2+} channels (SOCs) or I_{CRAC}	ER store depletion	Unclear
TRP channels	ER store depletion (partial) Alkaline extracellular pH (partial)	Apical & Basolateral
P2X receptor Ca^{2+} entry channels	Extracellular zinc and ATP	Apical & Basolateral
ECaC or CAT *(Related to TRPs)*	ER store depletion	Apical
Ca^{2+}-permeable non-selective cation channel (NSCC)	Stretch-activated	Apical

FIG.19D

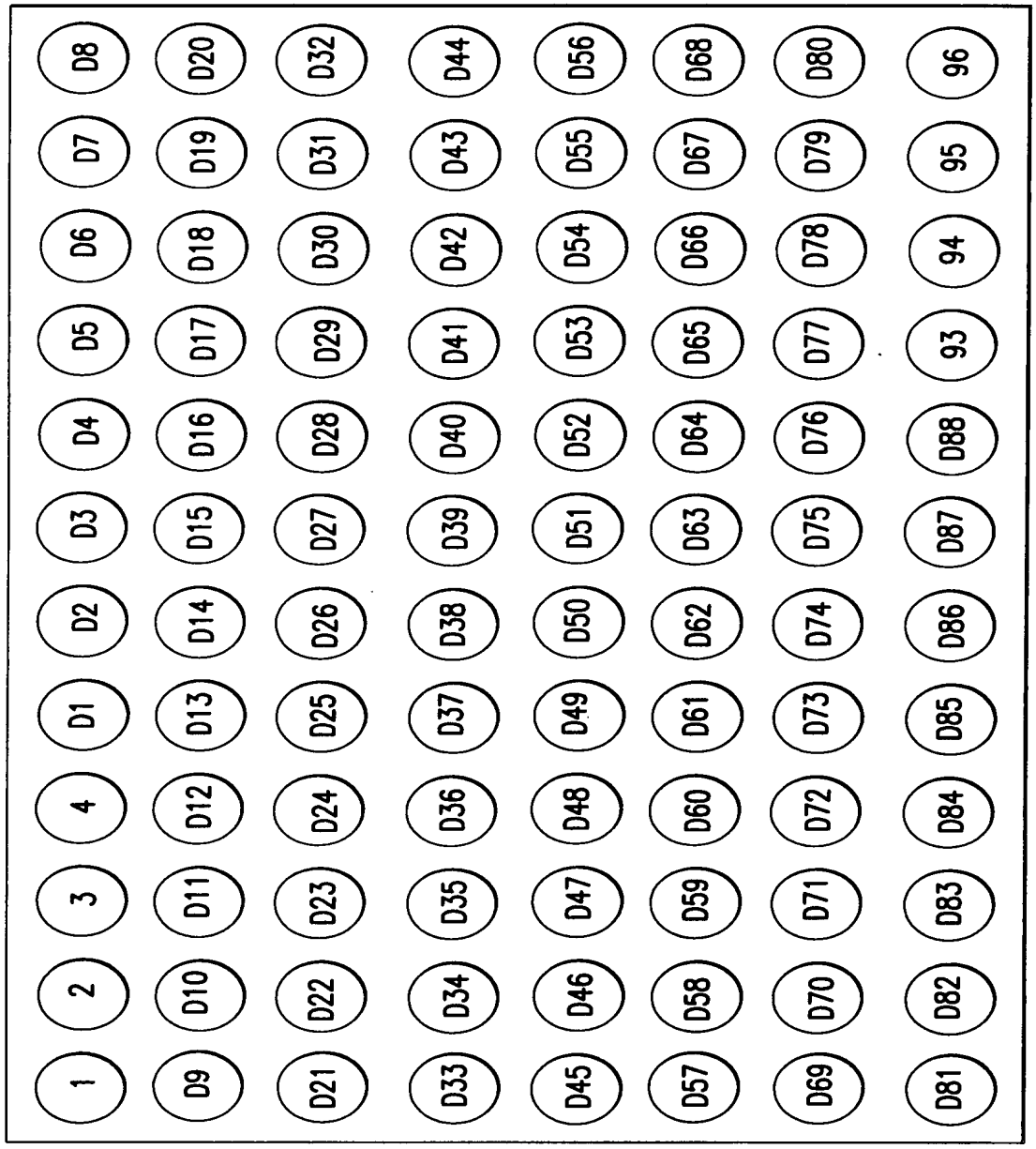

D8 D20 D32 D44 D56 D68 D80 96
D7 D19 D31 D43 D55 D67 D79 95
D6 D18 D30 D42 D54 D66 D78 94
D5 D17 D29 D41 D53 D65 D77 93
D4 D16 D28 D40 D52 D64 D76 D88
D3 D15 D27 D39 D51 D63 D75 D87
D2 D14 D26 D38 D50 D62 D74 D86
D1 D13 D25 D37 D49 D61 D73 D85
4 D12 D24 D36 D48 D60 D72 D84
3 D11 D23 D35 D47 D59 D71 D83
2 D10 D22 D34 D46 D58 D70 D82
1 D9 D21 D33 D45 D57 D69 D81

FIG.20A

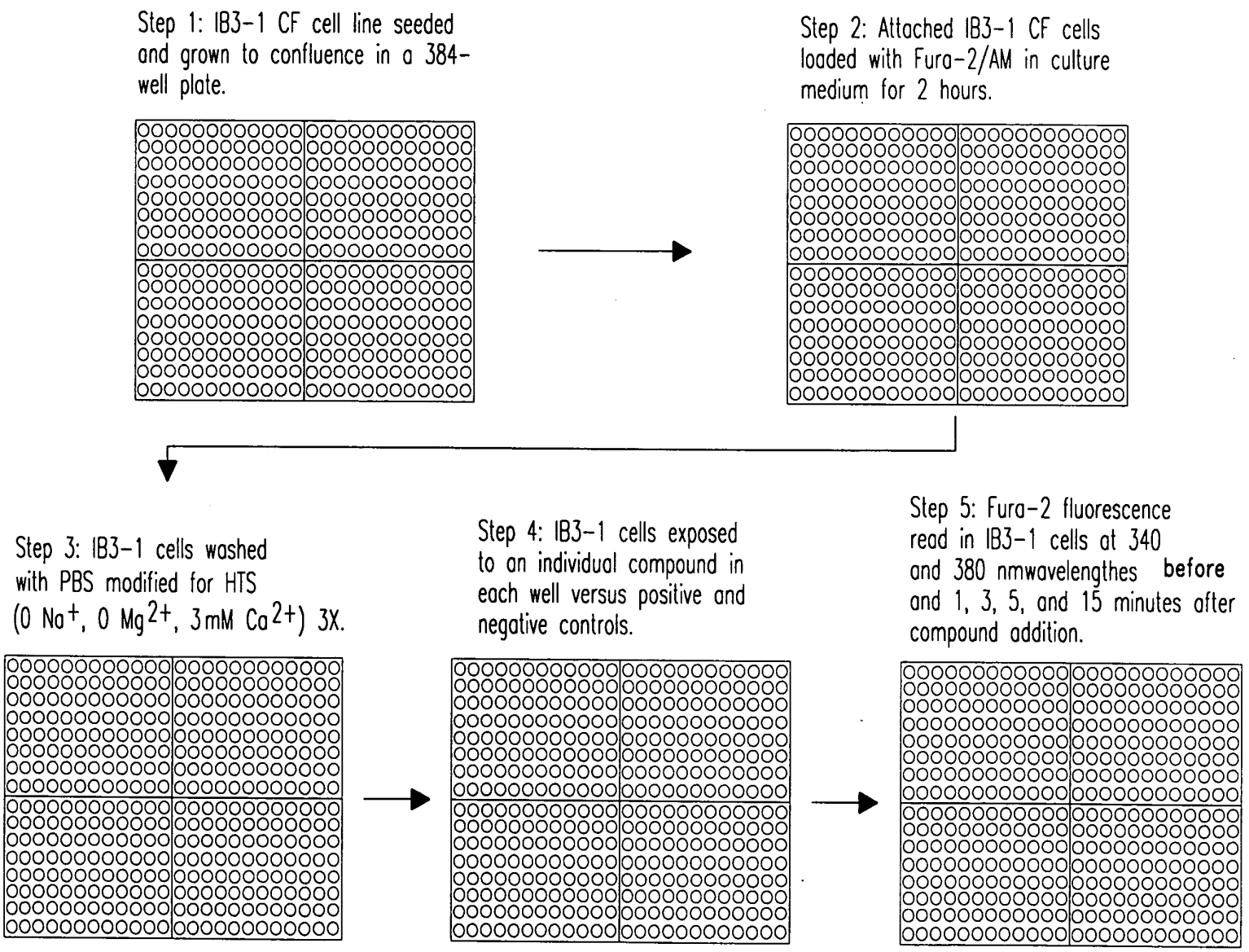

Step 1: IB3-1 CF cell line seeded and grown to confluence in a 384-well plate.
Step 2: Attached IB3-1 CF cells loaded with Fura-2/AM in culture medium for 2 hours.
Step 3: IB3-1 cells washed with PBS modified for HTS (0 Na^+, 0 Mg^{2+}, 3mM Ca^{2+}) 3X.
Step 4: IB3-1 cells exposed to an individual compound in each well versus positive and negative controls.
Step 5: Fura-2 fluorescence read in IB3-1 cells at 340 and 380 nmwavelengthes before and 1, 3, 5, and 15 minutes after compound addition.

FIG.20B

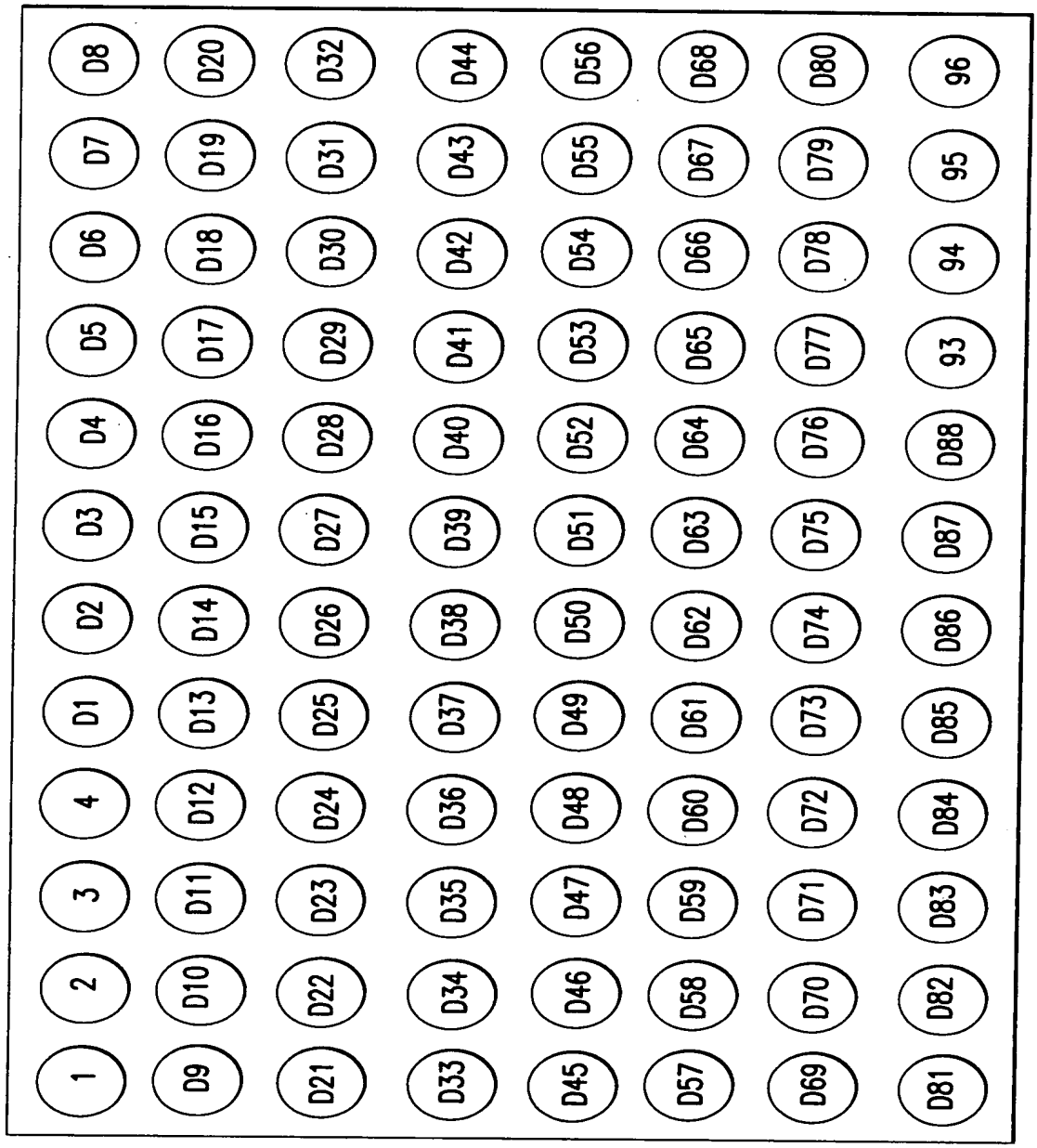

1
2
3
4
D1
D2
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
D13
D14
D15
D16
D17
D18
D19
D20
D21
D22
D23
D24
D25
D26
D27
D28
D29
D30
D31
D32
D33
D34
D35
D36
D37
D38
D39
D40
D41
D42
D43
D44
D45
D46
D47
D48
D49
D50
D51
D52
D53
D54
D55
D56
D57
D58
D59
D60
D61
D62
D63
D64
D65
D66
D67
D68
D69
D70
D71
D72
D73
D74
D75
D76
D77
D78
D79
D80
D81
D82
D83
D84
D85
D86
D87
D88
93
94
95
96

FIG.20C

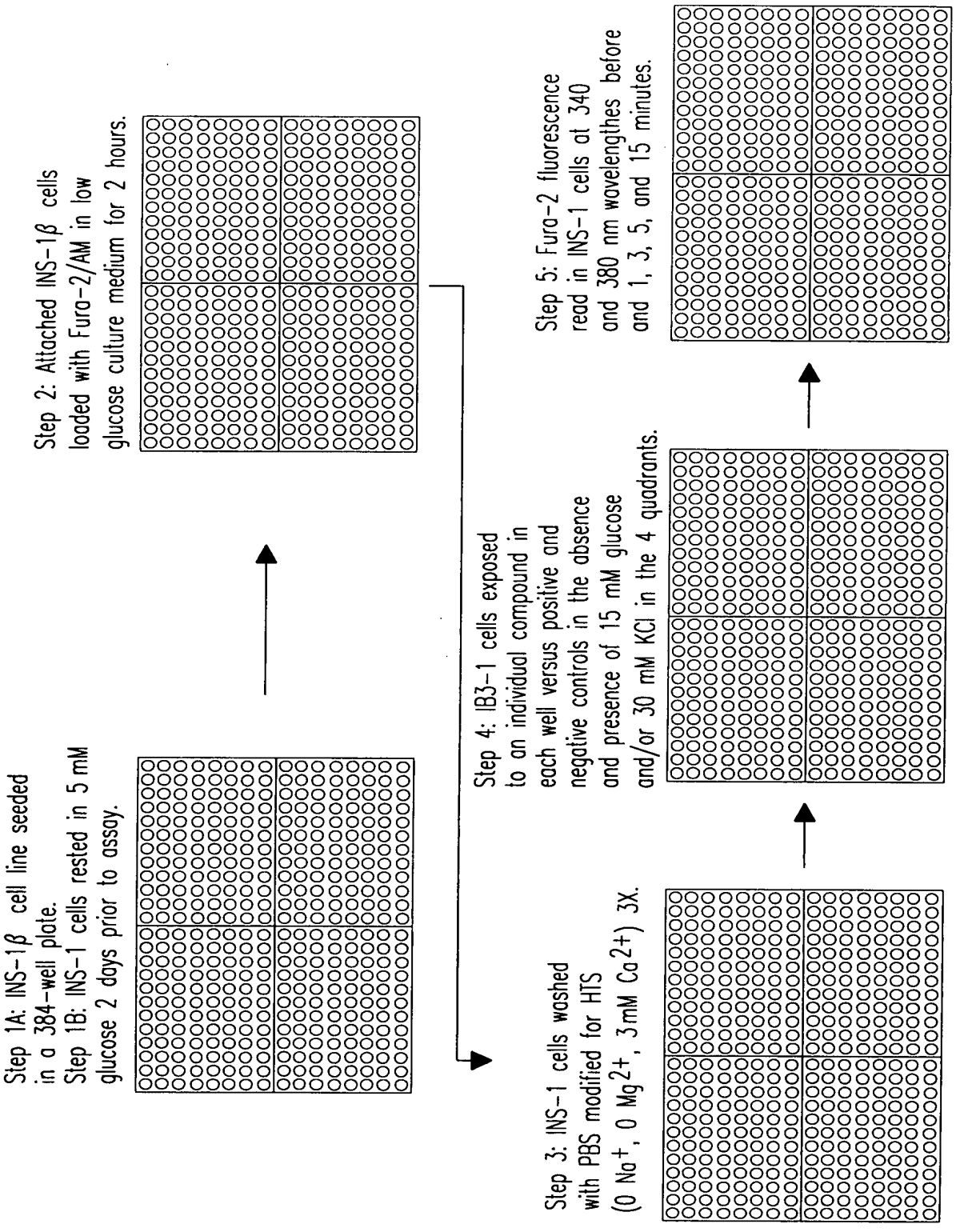

Step 1A: INS-1β cell line seeded in a 384-well plate.
Step 1B: INS-1 cells rested in 5 mM glucose 2 days prior to assay.
Step 2: Attached INS-1β cells loaded with Furo-2/AM in low glucose culture medium for 2 hours.
Step 3: INS-1 cells washed with PBS modified for HTS (0 Na^+, 0 Mg^{2+}, 3mM Ca^{2+}) 3X.
Step 4: IB3-1 cells exposed to an individual compound in each well versus positive and negative controls in the absence and presence of 15 mM glucose and/or 30 mM KCl in the 4 quadrants.
Step 5: Furo-2 fluorescence read in INS-1 cells at 340 and 380 nm wavelengthes before and 1, 3, 5, and 15 minutes.

FIG.20D

MAY 22 2007
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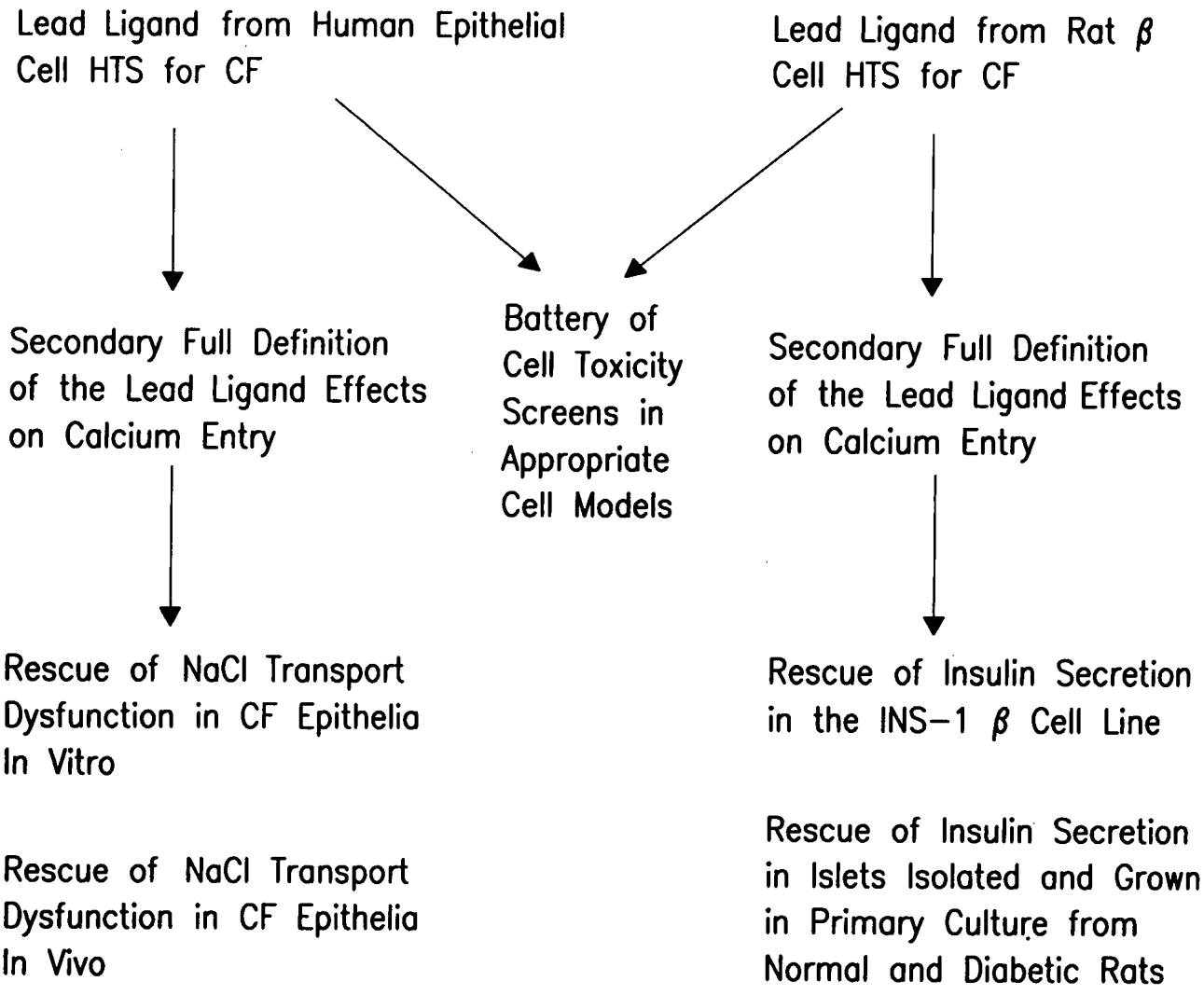

FIG.20E